BAKER'S YEAST β-KETO ESTER REDUCTIONS WHOLE CELL BIOCATALYSTS WITH IMPROVED STEREOSELECTIVITY BY RECOMBINANT DNA TECHNIQUES

By

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To all my loved ones family and friends
and in a very special note to Carlos

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BAKER S YEAST β KETO ESTER REDUCTIONS WHOLE CELL BIOCATALYSTS WITH IMPROVED STEREOSELECTIVITY BY RECOMBINANT DNA TECHNIQUES

By

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May 2000

Chairman Dr Jou D Stewart
Major Department Chemistry

Baker s yeast has been widely used for asymmetric synthesis of alcohols When a single enzyme dominates the reduction of a particular substrate, whole yeast cells can provide the chiral alcohol in high optical purity In many cases however multiple enzymes with opposing stereoselectivities are involved thereby diminishing the optical purity of the product This problem has restricted the utility of baker s yeast for the stereoselective synthesis of β hydroxy esters and α substituted β hydroxy esters

Isolated yeast β keto ester reductases provide highly stereoselective biocatalysts however their utility is hampered by the need for enzyme isolation and cofactor regeneration On the other hand, whole cells of baker s yeast provide a very handy and inexpensive reagent, but one that often exhibits incomplete stereoselectivity for reductions of these substrates The optimal biocatalytic solution to production of β-

hydroxy esters would be one that combines the advantages of the isolated enzyme [illegible] those of the whole cell system. The goal of this project has been to engineer yeast strains that mirror the stereoselectivities of the individual reductases.

In this work, the stereoselectivities of baker's yeast-catalyzed reductions of β-keto esters were manipulated by genetic design of yeast strains. Strains in which three β-keto ester reductases were either knocked out or over-expressed have been constructed. These first-generation strains provided good improvements in stereoselectivity; however incomplete stereoselectivity was still a common outcome of the reactions. In order to achieve higher levels of diastereo- and enantioselectivities, strains combining over-expression and knock-out strategies were designed. These second-generation strains resulted in better improvements in the stereoselectivity. One of these strains furnished *S* alcohols in > 98 % enantiomeric excess for all the tested ketones, nicely illustrating the potential of the engineered yeast strategy. In addition, other yeast strains selectively afforded the optically pure *syn* and *anti* diastereomers from reductions of appropriate β-keto esters.

These studies constitute the first time that a logical approach has been used to improve stereoselectivity in baker's yeast reductions and provided comparable or better improvements than those obtained with previous methodologies. The results obtained during this work also uncovered the participation of additional yeast reductases in reductions of β-keto esters. Some of these enzymes were identified based on their homology with known reductases and expressed in *Escherichia coli*. Whole cells of these engineered *E. coli* strains also acted as useful biocatalysts for the stereoselective reductions of β-keto esters.

CHAPTER1
BACKGROUND AND INTRODUCTION

Chirality in Organic Synthesis

Chirality plays a major role in molecular recognition since living systems interact with stereoisomers in decisively different manners. Drug synthesis strategies have been greatly affected by the control of chirality and examples of chiral drugs with one enantiomer being harmful to the human body are well known (Figure 1-1).[1] The earliest and most tragic example is thalidomide, a sedative administered as a racemate to prevent nausea during pregnancy. At the time, it was not known that the pharmacological activity resides in the (*R*) enantiomer (**5**) while the (*S*) counterpart (**6**) is teratogenic.[2,3] These examples have raised concerns related to the use of racemic drugs; consequently, current practice is geared towards the synthesis of optically pure materials in preference to racemates. This has led to an increased need for enantiopure compounds and driven the development of methodologies for controlling chirality.

First-generation enantioselective syntheses relied either on the transformation or derivatization of readily available members of the chiral pool – natural products such as amino acids, terpenes, carbohydrates, alkaloids, etc.[4,5] – or resolution of racemates. Later efforts have focussed on increasing the access to enantiomerically pure compounds from prochiral precursors.[3,6] These efforts have provided a group of chemical as well as biological processes that make a variety of optically active building blocks available for

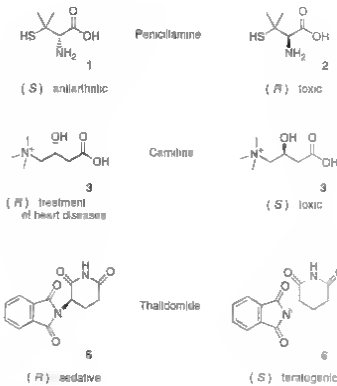


Figure 1 1 Biological effects of some drug enantiomers

asymmetric total syntheses Current work in this field is aimed at achieving better stereoselectivity, higher catalytic efficiencies and more environmentally friendly methodologies, as well as increasing the diversity of available chiral starting materials Because chiral alcohols are a particularly valuable class of chiral starting materials a great deal of effort has been made to provide these synthons and they have been the focus of this work

Optically Pure β-Hydroxy Esters as Building Blocks for Chiral Synthesis

Optically active alcohols and hydroxy esters constitute a particularly versatile class of chiral building blocks since a wide variety of further chemical derivatizations is possible [6,7] A broad array of natural and unnatural products has been synthesized from chiral alcohols in recent years [6] Chiral β hydroxy esters have also been used for total synthesis of natural products [7-9] among those the preparation of several optically active pheromones as those presented in Figure 1 2 (**7**-**13**) have been reported [10-17] These chiral building blocks have also found application in the synthesis of important drugs and some examples are shown in Figure 1 2 Carnitine (**15**) used in the treatment of heart disease and GABOB (**16**)–an antiepileptic drug–can be synthesized from a common chiral chloro hydroxy ester [18 19] In addition chiral β-hydroxy esters have also been used for the synthesis of several antibiotics like the polioxomacrolide derivatives [20] β lactam antibiotics [8,9] and griseoviridin–a member of the streptogramin antibiotic family [21]

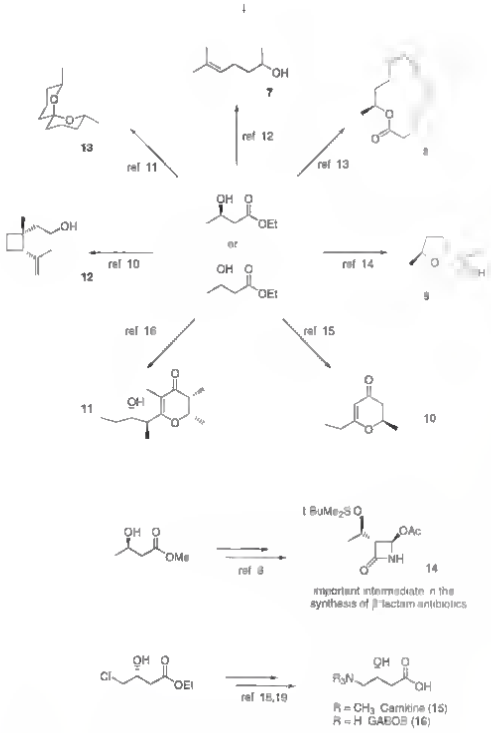


Figure 1 2 Applications of chiral β hydroxy esters in asymmetric synthesis

Synthesis of Chiral β-Hydroxy Esters: Chemical Methodologies

Attempts to develop practical routes to chiral β-hydroxy esters have explored both chemical and biological strategies and these will be considered separately. Chemical methods for synthesis of these compounds are summarized in Figure 1-3. The most common strategy involves asymmetric reduction of β keto esters. Although the use of simple borohydride reagents such as $NaBH_4$ or LAH has not proven useful, modifications to increase the stereoselectivities of these processes have been developed. Hydrogenations using Ru based catalysts have also been very successful for the synthesis of several β hydroxy esters. Despite success in delivering α unsubstituted β hydroxy esters, few methods have been reported for the synthesis of chiral α substituted β hydroxy esters. This is unfortunate since these synthons are of particular interest for their densely packed functionality with two stereocenters. Chemical strategies for these building blocks have included aldol condensations and stereoselective alkylations of β-hydroxy esters. Although some successful applications have been reported, a general route to this class of compounds remains to be developed.

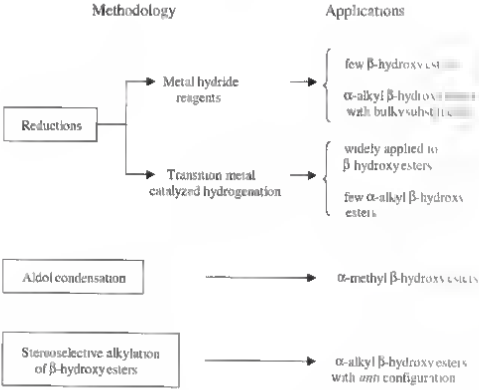


Figure 1-3 Chemical methods for the synthesis of chiral β hydroxy esters and α alkyl β-hydroxy esters.

Reductions

Asymmetric reduction of the corresponding β-keto esters both by chemical and enzymatic reagents has been the most common route to β hydroxy ester building blocks. Several recent reviews have described the state of the art in chemical reduction of prochiral ketones including β keto esters.[6,22-25] The description of these methodologies will be divided into sections covering metal hydride reagents and catalytic hydrogenation the latter being the most extensively explored method for reduction of β-keto esters.

Reductions with metal hydrides

The use of metal hydride reagents for the stereoselective reduction of β-keto [illegible] has been limited to the use of borohydride reagents such as $LiBH_4$ and $Zn(BH_4)_2$ and relatively few applications have been reported. Stereoselection has been controlled by either adding chiral auxiliaries [26] or exploiting the innate ability of β keto esters to coordinate transition metals [20,27,28]. Although good enantio- and diastereoselectivities have been obtained for some substrates, all these methods use stoichiometric reagents and catalytic processes are much more desirable nowadays.

Use of chiral auxiliaries

Since the pioneering work of Bothner-By [29] the use of chirally modified reducing reagents has been a focus of much attention. However, successful application of this type of reagents to asymmetric reductions of β keto esters has been rather limited. A complex prepared from N, N' – dibenzoyl cysteine and t-butyl alcohol with $LiBH_4$ reduced aromatic β keto esters with up to 91 % enantiomeric excess and constitutes the only relevant example [26].

$Zn(BH_4)_2$ as reducing agent

Reduction with a metal hydride whose metal possesses coordinating ability has been shown to induce stereoselection for reduction of α-substituted β-keto esters. This method is only suitable for substituted β keto esters and the diastereoselectivity obtained varied from 1 to >98 % depending on the substrate (Figure 1-4) [20]. Attempts to apply this methodology to the enantioselective reduction of non-substituted β keto esters have also been reported.[27] In such cases the stereoselection is induced by the presence of a bulky

chiral alcohol (β-pinene derivatives) in the ester moiety (Figure 1-5). The enantiom[illegible] excess obtained varies from 2-70 % depending on the bulkiness of the chiral alcohol

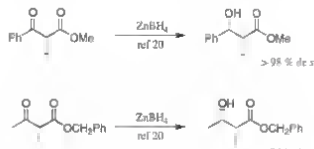


Figure 1-4 $ZnBH_4$ mediated diastereoselective reductions of α-alkyl β keto esters

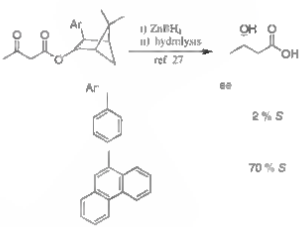


Figure 1-5 $ZnBH_4$ mediated enantioselective reductions of β keto esters induced by β pinene derivatives

Lewis acid mediated hydride reduction

Reductions by borohydride reagents have been showo to proceed with different stereoselectivities depending on the nature of an added Lewis acid [28]. The different chelating ability of $TiCl_4$ and $CeCl_3$ was used to induce different diastereoselection in the reduction of α-substituted β-keto esters (Figure 1-6). Very good diastereoselectivities were obtained, however, high stereoselectivity required the presence of bulky substituent to guarantee the appropriate transition state. Moreover, while this strategy controls the diastereoselectivity, the enantioselectivity is determined by the optical purity of the starting material.

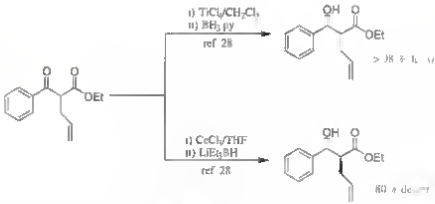


Figure 1-6. The diastereoselectivity of α-alkyl β-keto ester reductions by borohydride reagents depends on the chelating properties of the associated Lewis acid.

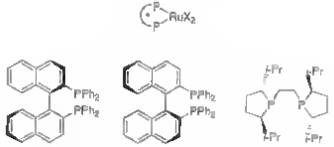
Transition metal-catalyzed hydrogenations

The development of chiral metal complexes for asymmetric hydrogenation has provided some highly efficient catalysts. Although they are required in very small

quantities, the cost associated with the use of precious metals like Ru and Rh can make the process expensive on large scale. Much work has been done in the development of these catalysts and several applications to the reduction of β-keto esters have been reported.[23,25,30]

Highly enantioselective hydrogenation of α- or β-keto esters has been achieved with some Ru catalysts (Figure 1-7). While the BINAP-Ru dicarboxylate complexes were totally ineffective, the halogen-containing complexes RuX_2(BINAP) provided useful catalysts (Table 1-1, entries 1-4).[30] Unfortunately, these catalysts require high temperatures and pressures, or acid co-catalysts for acceptable catalytic rates. However, milder conditions have been recently reported (Table 1-1, entries 5-7).[31] Bis-(phospholane) ligands such as BPE also achieve highly enantioselective Ru-catalyzed hydrogenation of β-keto ester under milder conditions (Table 1-1, entries 8-10).[24,3

Ru(II) catalysts used in asymmetric hydrogenation of β-keto esters



(*R*)-BINAP (*S*)-BINAP (*R,R*)-*i*Pr BPE

Figure 1.7 Asymmetric hydrogenation of β-keto esters with Ru(II) based catalysts

Despite the high stereoselectivities that Ru(II) catalysts show towards β-keto esters few successes have been achieved for reductions of α substituted β keto esters and some examples are summarized in Table 1.2. The efficiency and sense of the enantio- and diastereoselection for α-alkyl β-keto esters is highly influenced by substrate structure and reaction conditions. Simple systems such as ethyl 2-methyl acetoacetate exhibited essentially no diastereoselection (Table 1.2 entry 1).[32,33] Some acyclic β-keto esters with bulky substituents yielded the *syn* optically-active hydroxy esters (Table 1.2 entry 2) while *anti* products were obtained from cyclic β keto esters (Table 1.2 entry 3).[34]

Table 1-1 Asymmetric hydrogenation of β keto esters with Ru(II) based c[illegible]

entry	R_1	R_2	catalyst	experim. cond.	% yield	% ee	[illegible]	[illegible]
1	CH_3	CH_3	$RuCl_2$[(*R*) BINAP]	30 °C/ 100 atm. H	96	>99	*R*	[illegible]
2	CH_3	CH_3	$RuCl_2$[(*S*)-BINAP]	30 °C/ 83 atm H_2	97	>99	*S*	[illegible]
3	C_2H_5	CH_3	$RuBr_2$[(*R*) BINAP]	30 °C/ 92 atm H_2	99	100	*R*	30
4	CH_3	C_2H_5	$RuCl_2$[(*R*) BINAP]	30 °C/ 103 atm. H_2	99	99	*R*	30
5	CH_3	CH_3	$RuBr_2$[(*S*)-BINAP]	R.T./ 1 atm H_2	80	97	*S*	31
6	C_2H_5	CH_3	$RuBr_2$[(*S*) BINAP]	50 °C/ 1 atm H_2	100	99	*S*	31
7	C_2H_5	CH_3	$RuBr_2$[(*R*) MeO Biphep]	50 °C/ 1 atm H_2	100	99	*R*	31
8	CH_3	CH_3	(*R R*)-*i* Pr BPE Ru	35 °C/ 60 psi H		99.3	*S*	32
9	CH_3	t Bu	(*R,R*) *i*-Pr BPE Ru	35 °C/ 60 psi H_2		99.4	*S*	32
10	C_2H_5	CH_3	(*R R*) *i* Pr BPE Ru	35 °C/ 60 psi H		98.6	*S*	32

Table 1-2 Diastereoselectivity of Ru(II) catalysts for reduction of α-substituted β-keto esters

entry	Substrate	product	Catalyst	*syn anti*		
1			$RuBr_2$(*R*)BINAP (*R*,*R*) *i*-Pr-BPE-Ru	51 49 58 42		
2			$RuBr_2$(*R*)BINAP	99 1		
3			$RuBr_2$(*R*)BINAP (*R*,*R*) *i*-Pr-BPE-Ru	99 1 96 4		

Ru-based catalysts provide good methodologies for obtaining β hydroxy esters of high optical purity however less progress has been made towards the synthesis of α substituted β hydroxy esters A major problem with this methodology is the use of expensive catalysts, precious metals and chiral ligands Attempts to use complexes with cheaper metals such as Co and easily available nitrogen-containing ligands resulted in low ee's for the conversion of β keto esters.[34] These problems have hampered the use of these catalysts for practical purposes

Aldol Condensation

In spite of the successful application of the aldol condensation to other synthons it does not provide a practical route to optically pure α alkyl β hydroxy esters since successful applications have been limited to formation of α-methyl-β-hydroxy esters In addition control of both enantio and diastereoselectivity required the use of chiral

enolates in stoichiometric amounts. Use of chiral reagents usually results in costly processes, particularly when non-catalytic methods are involved.

The discovery that the stereochemistry of an aldol condensation can be controlled effectively through the use of preformed enolates led to new applications of this reaction.[35] The reaction between a prochiral enolate and an aldehyde can result in two diastereomeric β hydroxy ketones and the relative stereochemistry of the aldol product is controlled by that of the enolate from which it is derived. Provided the group attached to the oxygen bearing carbon of the enolate is bulky, *Z* and *E* enolates resulted in formation of *syn* and *anti* aldols respectively.

Diastereoselective synthesis of *syn* α alkyl β hydroxy acids was first achieved by condensation of the α-trimethylsilyloxy ketone enolate **17** with an aldehyde, followed by treatment with periodic acid (Figure 1-8).[36] Synthesis of *anti* α-alkyl β hydroxy esters could in principle result from direct condensation of ester enolates with aldehydes since esters tend to form the *E* enolate upon deprotonation with LDA. Unfortunately, the enolates of normal alkyl esters show essentially no stereoselection in their reaction. On the other hand, by using particular phenyl esters, good diastereoselectivity could be achieved[37] and one example was the reaction of the ester enolate **18** with isobutyraldehyde (Figure 1-8).

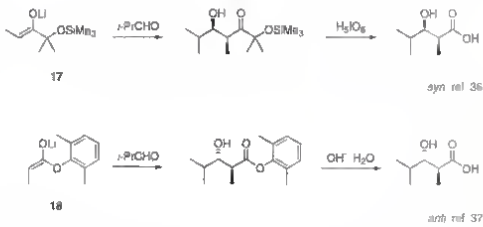
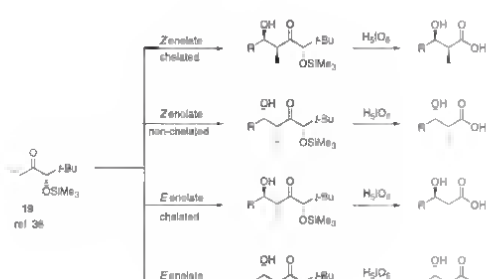


Figure 1-8 Stereoselective aldol condensations in the construction of *syn* and *anti* β hydroxy esters

In all these examples condensation favored one diastereomer, but there was no control on the enantioselectivity of the reaction Control of both enantio and diastereoselectivity required the use of a chiral enolate as starting material A chiral ketone can be converted into each of the four possible diastereomers by regulating the stereochemistry of enolate formation and by selecting whether it reacts on its *si* or *re* face The stereochemistry of the enolates can be regulated by appropriate choice of base whereas facial diastereoselection can be regulated by addition of chelating or non chelating metals This strategy was applied using ketone **19** as starting material different combinations of base and metal resulted in formation of the four possible stereoisomers as depicted in Figure 1-9 [35 38] Treatment with periodic acid yielded the corresponding α methyl β-hydroxy carboxylic acid The Evans asymmetric aldol reaction also provides a highly stereoselective methodology that usually results in production of the *syn* diastereomer [39] Chiral imides such as **20** have very high diastereofacial preferences in their

reactions with aldehydes Hydrolysis of the resulting aldol provides the α alkyl β hydroxy carboxylic acid.

New conditions for the boron mediated aldol condensation of simple carboxylic esters have recently corrected the long term misconception that these compounds could not be enolized under common conditions (dialkyl boron triflate and an amine) Appropriate choice of the amine and the dialkylboron triflate was essential for successful synthesis of α-methyl β hydroxy esters from alkyl ester propionates [40] The *syn anti* selectivity is sensitive to the alcohol residue of the ester and the choice of enolization reagents These findings led Masamune to investigate the stereoselectivity of the aldol reaction of various chiral propionate esters resulting in *anti* and *syn* selective aldol reagents compounds **21** and **22** respectively (Figure 1-9) [41,42]



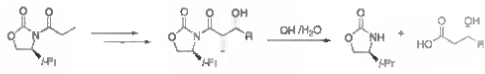


Figure 1.9. Diastereofacial selection in aldol condensations with a chiral enolate.

Although the combination of an appropriate chiral starting material and experimental conditions provided the four possible diastereomers of α-substituted β hydroxy esters these reactions require suitable chiral metal enolates in stoichiometric

amounts and in many cases an additional reaction to achieve the desired product. Furthermore, applications of the aldol condensation to the synthesis of optically pure β-hydroxy esters have been limited to formation of α-methyl β-hydroxy esters and this does not constitute a general methodology for the construction of chiral α-alkyl β-hydroxy esters.

Stereoselective Alkylation of β-Hydroxy Esters – Frater Alkylation.

A second methodology based on C-C bond-forming reactions has been successfully applied to the synthesis of *anti* α-alkyl β-hydroxy esters. Stereoselective alkylation of optically pure β-hydroxy esters has provided these synthons with good optical purities (Figure 1-10).[43] In these reactions, the alkylation occurs on the less hindered face to yield the *anti* diastereomer. Although the alkylation occurred with high stereoselectivity, the enantioselectivity is limited by the optical purity of the starting material. This strategy is also limited to production of the *anti* diastereomer and to those optically pure β-hydroxy esters available by other means.

R	% yield	Alk. cont	% de
CH_3	68	2*S*, 3*S*	91
Allyl	75	2*S*, 3*S*	90
Benzyl	82	2*S*, 3*S*	84

Figure 1-10. Production of *anti* ethyl 2-alkyl 3(*S*)-hydroxanoates through Frater alkylation.

Synthesis of Chiral β Hydroxy Esters: Biocatalysis

The application of biocatalysts as chiral reagents has become increasingly important in contemporary organic synthesis. Enzyme methods usually require mild conditions, which minimizes problems of undesired side reactions such as decomposition, isomerization, racemization, epimerization and rearrangements. Enzymes are often not limited to their natural substrate and some accept a large variety of man-made, unnatural substances. Finally, once the reactions are completed biocatalysts are completely degraded in the environment, thereby providing an environmentally friendly alternative to classical methodologies. Given these advantages, it is not surprising that enzymatic routes to β-hydroxy esters have been explored extensively.

With the exception of some lipase mediated resolution of the acetylated alcohols [44-46] or the hydrolysis of naturally occurring polymers such as poly hydroxy butyrate [47], most biocatalytic routes to β hydroxy esters have focused on stereoselective reduction of β-keto esters. A number of biocatalytic reagents, encompassing both purified enzymes and whole microbial cells, have been investigated [48-50]. Even though some other microorganisms have been shown to reduce β-keto esters, most studies have been focussed on reductions with baker's yeast or reductases isolated from this microorganism. Because of the large amount of literature in this field, the state of the art on the use of baker's yeast for synthesis of β hydroxy esters is considered in a separate section.

Purified Enzymes

A broad range of prochiral ketones can be reduced stereoselectively to give chiral secondary alcohols, and various alcohol dehydrogenases have been used as chiral

catalysts for these conversions [3,48] In most cases the stereochemical course of the reaction may be predicted from the Prelog s rule (Figure 1-11) [51] although dehydrogenases that lead to the formation of anti-Prelog alcohols are known [1]

Figure 1-11 Prelog s rule for the asymmetric reduction of ketones

A few reductases purified from microorganisms other than baker s yeast have been used to synthesize β- hydroxy esters and some representative examples are summarized in Figure 1-12 Alcohol dehydrogenases isolated from *Geotrichum candidum* (GCDH)[52] and the thermophilic microorganism *Thermoanaerobium brockii* (TADH)[51] have been used for the asymmetric reduction of β- keto esters [49,54-57] While these two enzymes yielded the (*S*) alcohol for the reduction of ethyl 4-chloro acetoacetate an aldehyde reductase isolated from *Sporobolomyces salmonicolor* provided the opposite enantiomer [58] The latter has been further characterized for reduction of a number of halogenated β- keto esters, along with a carbonyl reductase from *Candida magnoliae* [59] Few enzymes have been used for the reduction of α-substituted β- keto esters (Figure 1-12) A commercially available reductase from *Pseudomonas lemoignei* exhibited broad substrate specificity and yielded the *syn* (2*S* 3*R*) isomer in good to high optical purity [60] An enzyme that catalyzes the reduction of ethyl α-methyl acetoacetate has been recently

isolated from *Klebsiella pneumoniae* and has been shown to reduce several β k-

esters [81]

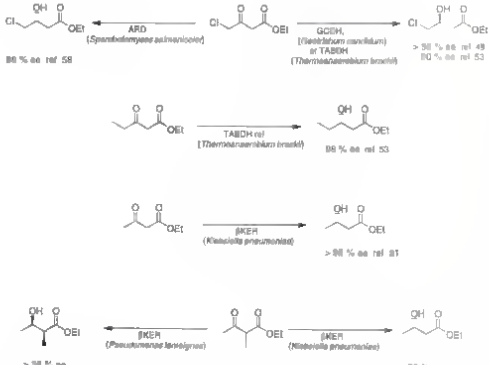


Figure 1-12 Synthesis of optically pure β-hydroxy esters by isolated enzymes

The major advantages of using isolated enzymes instead of the whole cell biocatalyst are related to their specificity and high stereoselectivity. Enzymes are very specific for a type of functional group and reaction and they are usually highly stereoselective. Therefore the use of isolated enzymes avoids undesired side reactions that may occur in a whole cell biocatalyst due to the presence of competing enzymes. However, there are major drawbacks with the use of isolated enzymes and these are

usually related with the protein isolation process and cofactor requirement. Unless a crude extract can be used, enzyme isolation is an expensive procedure. Some enzymes are stable and can even be reused, however this has not been the case for reductases. Furthermore, reductases require redox cofactors, which donate the hydride equivalents for reduction. For the majority of these enzymes, nicotinamide adenine dinucleotide (NADH) or the phosphorylated derivative (NADPH) are required. These are relatively unstable molecules and prohibitively expensive if used in stoichiometric amounts. Thus, a large effort in this field has been devoted to the development of efficient recycling systems.[62] However, the recycling of these cofactors is still problematic and constitutes an economic barrier for large scale reactions.[3]

Whole Cell Systems

Issues of enzyme isolation and cofactor recycling are avoided when whole microbial cells are used as biocatalysts since both are provided by growing cells. The use of whole cells also allows biocatalysis to be employed even when the enzyme responsible for a particular conversion is not known. Several microorganisms have been found to be useful for the enantioselective reductions of β keto esters (Figure 1.13) and their applications have been reviewed.[48,50,63] While most examples have been limited to the use of yeast or fungi, some bacterial strains [64] and the algae *Chlorella* [65] have also been shown to reduce these compounds.

Some useful applications of whole cell mediated reductions are summarized in Figure 1.13. The absolute configuration of the alcohol product is determined by the nature of the microorganism and by the substrate.[48,50] For some substrates such as **23**-**26** (Figure 1.13), β hydroxy esters of either configuration can be obtained by appropriate

choice of microorganism [41 5 66-70] The screening of several yeast strains from the genera *Kloeckera*, *Hansenula*, *Candida*, and *Torulopsis* for enantioselective reduction of β keto estet **25** resulted in two strains *Candida boidonii* and *Hansenula anomala* that produced each enantiomer with good enantioselectivity [69] Similar studies preceded the identification of the different strains represented in Figure 1 13 A major disadvantage of this approach is that a microorganism that presents high enantioselectivity for a particular substrate may not show the same for others, and a new screening process may be necessary for each biotransformation Although several microorganisms have been reported for reduction of particular substrate, the screening process is a tedious task In one example Sugiyama screened 450 strains of bacteria for the enantioselective reduction of ethyl α-methyl acetoacetate and identified a *Klebsiella pneumoniae* strain as a suitable reagent [64]

The preparation of optically pure α-alkyl-β-hydroxy esters represents an even more challenging synthetic problem and it is not easily accomplished by the reduction of the racemic ketone *Geotrichum* sp has been characterized for the reduction of several β keto esters and diketones including the reduction of α substituted β keto esters [7 7] This fungus showed great enantioselectivity but only moderate diastereoselectivity a similar situation to the use of baker s yeast.[43 72] *Mucor javanicus* was found to yield the opposite diastereomer than baker s yeast for the reduction of **27** [70] however the same problem of incomplete diastereoselectivity was also found for this strain

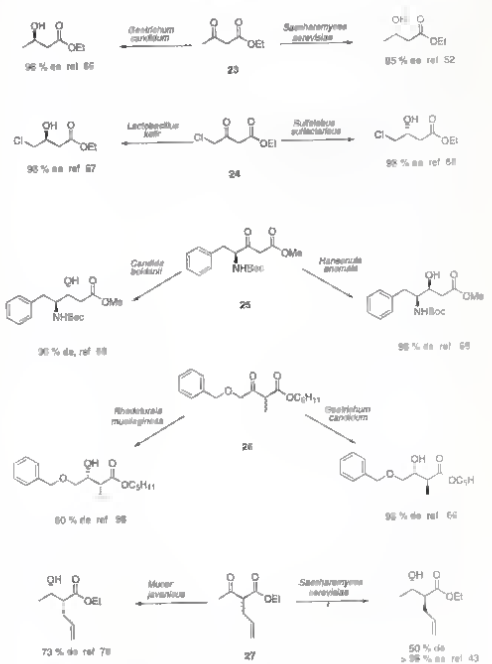


Figure 1-13 Whole cell-mediated synthesis of β hydroxy esters

Despite some successful examples of whole cell-mediated stereoselective syntheses of β hydroxy esters the utility of the organisms is limited to particular substrates and the microbe cannot be regarded as a generally useful enantioselective reagent. This can be due to individual enzymes with poor stereoselectivity or in the presence of other enzymes that compete for the substrate lowering the stereoselectivity of the reaction

Recombinant Microorganisms

The combination of molecular biology techniques with genome sequence data from different organisms has opened a new era in the development of biocatalysts. The expression of natural or mutant enzymes suitable for particular synthetic applications in recombinant microorganisms provides an efficient source of enzymes and/or whole cell biocatalysts. This strategy has already been applied to whole cell biocatalysis using baker's yeast and *E. coli* as host microorganisms [73,74]. A recombinant *E. coli* strain expressing an aldehyde reductase from *Sporobolomyces salmonicolor* has been used for reduction of ethyl 4-chloro acetoacetate to the corresponding (*R*) alcohol in 91 % ee [75]. Whole cells of the recombinant bacteria were used in a two-phase system that also contained an NADPH regeneration system. In this approach the pre-grown cells acted only as a source of the reductase enzyme [76]however growing cells can also supply enough cofactor for biotransformations. Because enzyme expression in *E. coli* is generally applicable this strategy offers a simple way to utilize reductase enzymes from a diverse array of organisms.

Baker s Yeast in Organic Synthesis

Although the concept of enzyme applications to asymmetric synthesis has been long recognized, the inclusion of biocatalysts as part of the repertoire of reagents in organic synthesis has been somewhat restricted This reluctance has been well summarized by Kurt Faber

> Any exponents of classical organic chemistry will probably hesitate to consider a biochemical solution for one of their synthetic problems This would be due, very often to the fact that biological systems would have to be handled. When growth and maintenance of whole microorganisms is concerned, such hesitation is probably justified.[3]

This statement probably reflects the major reason that baker s yeast continues to be the most popular and well-characterized whole-cell biocatalyst for asymmetric organic synthesis. Baker s yeast is particularly well suited for organic chemistry since it is readily available inexpensive, non-pathogenic and reactions do not require any microbiology expertise A large variety of reactions including ester and amide hydrolysis aldol and acetoin condensations polyene cyclization and carbonyl and olefin reductions have been catalyzed by baker s yeast [49 77] Asymmetric reduction of carbonyl compounds has been by far the most common application of this biocatalyst. The catalytic repertoire of baker s yeast has been subject of several comprehensive review articles [49,50 77 79] and practical procedures for yeast biotransformations have been reported [52,80]

Reduction of Carbonyl Compounds with Baker s Yeast

The reducing capacities of yeast have attracted the interest of modern organic chemists in the preparation of chiral secondary alcohols The observation that the addition of sulfur in fermenting yeast resulted in the formation of H_2S was probably the first

literature account of the reducing capacity of yeast.[81] The reduction of carbonyl groups was first shown with aldehydes and then applied to ketones.[82] Since then, the baker's yeast-mediated reduction of carbonyl compounds for the preparation of chiral secondary alcohols has become an established procedure and several examples are illustrated in Figure 1.14.[83-90] Reduction of ketones, diketones, α-substituted ketones, α- and β-keto esters, including both cyclic and open chain compounds have been reported, and in many cases the products showed high optical purity.

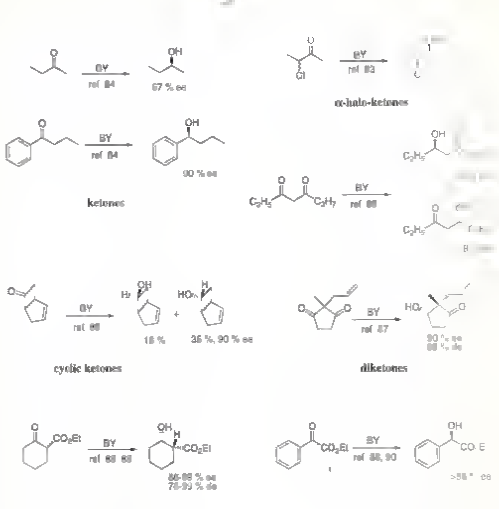


Figure 1-14 Some applications of baker s yeast (BY) in the asymmetric reductions of carbonyl compounds

The first industrial application of baker s yeast reductions was the synthesis of chiral δ-lactones used in the food industry [91] Industrial preparation of trimegestone (18) via the baker's yeast catalyzed reduction of the corresponding triketone (17) has been recently reported (Figure 1-15) [92] The fact that yeast reductions have been applied in an industrial scale adds significance to this biocatalyst

Figure 1-15 Industrial application of baker s yeast to the production of trimegestone

Probably the most common biotransformation by baker's yeast is the reduction of β-keto esters first described in 1918 [93] The best-known example, reported in an *Organic Synthesis* preparation is the reduction of ethyl acetoacetate to yield ethyl (*S*)-(+) 3 hydroxy-butanoate with 85 % enantiomeric excess [52] Several β-keto esters are readily reduced by yeast to yield chiral β-hydroxy esters however the enantioselectivity varies depending on the substrate (Figure 1-16, Table 1-3) The reason for this divergent behavior is not due to an alternative fit of the substrates in a single enzyme but rather to the action of several oxido-reductases possessing opposite stereoselectivities and overlapping substrate specificity [94]

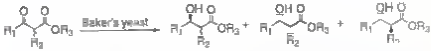


Figure 1-16. Baker s yeast mediated reductions of open chain β-keto esters

Table 1 3 Stereoselectivity of baker s yeast-mediated reduction of β keto esters (Figure 1 16)

Substrate (30)			% yield	Abs Conf	% ee	% de	Ref
R_1	R_2	R_3					
CH_3	H	CH_3	23	*S*	87		95
CH_2CH_3	H	CH_3	-	*R*	5		96
$ClCH_2$	H	CH_2CH_3		*S*	55		18
CH_3	H	CH_2CH_3	76	*S*	85		52
CH_2CH_3	H	CH_2CH_3	61	*R*	4		97
n C_3H_7	H	CH_2CH_3	-	*R*	86		98
CH_3	CH_3	CH_2CH_3	75	*syn* 2*R* 3*S*	>98	66	99
CH_3	CH_2CH_3	CH_2CH_3	80	*syn* 2*R* 3*S*	>98	32	100
CH_3	Allyl	CH_2CH_3	71	*anti* 2*S* 3*S*	>98	40	100
CH_3	Propargyl	CH_2CH_3	66	*syn* 2*R* 3*S*	>98	36	101

β Keto Ester Oxido-Reductases in Baker s Yeast

As is apparent from the data in Table 1 3 reductions of several β-keto esters resulted in mixtures of enantiomers If these β-hydroxy esters were due to a single enzyme it would require the enzyme to interact with both faces of the carbonyl group A second possibility was that yeast contained more than one oxido-reductase which generated carbinols of opposite configurations but at different rates The first evidence that the low stereoselectivity of β-keto ester reductions by baker s yeast was associated with the presence of more than one oxido-reductase was presented by Sih and co workers,[18] who demonstrated that the optical purities of several alcohol products varied with changes in substrate concentration [18,102]

Biochemical[103] and genome analysis[104] of baker s yeast has showo that a number of enzymes may be involved in the reduction of β keto esters Several groups have reported the isolation and characterization of β keto ester-reducing enzymes (Table 1–4) [60 74 105 107] Most of the enzymes were purified from the cytosolic fraction of yeast and it appears that different groups may have identified identical enzymes. In nearly all cases the physiological substrates for these enzymes are not known instead they have been purified on the basis of activity towards a specific ketone These practices have led to *ad hoc* naming of yeast reductases that often derive from the order of elution from specific chromatographic columns or reflect the nature of the assay substrate used during the purification

Three competing NADPH dependent β keto ester reductases capable of reducing 4 chloro acetoacetate esters with high enantioselectivity were first isolated from the cytosolic fraction of baker s yeast by Sih and co-workers [74] One of the enzymes (D enzyme-2) was assigned as the fatty acid synthetase (FAS) complex and was the predominant activity forming the D-carbinolic product. The two other enzymes referred to as D-enzyme-1 and L-enzyme, reduced β keto esters to yield the D and the L carbinols respectively The same group later reported another L-enzyme that produced also L-carbinols but shown opposite diastereoselection than the previous L enzyme [80] Hendlin et al [107] also reported two β keto ester reductases that were named (*R*) and (*S*) enzymes accordingly to the configuration of their products The (*R*) enzyme was considered to be identical to a subunit of the FAS complex The (*S*) enzyme however was distinct from those previously reported in differed in molecular mass and substrate specificity being able to enantioselectively reduce 4-oxo and 5-oxo acids and esters as

well as β-keto esters. Furuichi and co-workers purified an enzyme that catalyzed the reduction of benzyl-2-methyl-3-oxo-butyrate to yield preferentially the *syn* (2*R*,3*S*) diastereomer.[106] Nakamura and co-workers isolated four β-keto ester reductases from the cytosolic fraction of baker's yeast.[105] The two D-enzymes described by Nakamura seem identical to those described previously by Sih[94] and Heidlas.[107] Among the so-called L-enzymes, the E2 (also L-enzyme-2 and YKER VI) reported by Nakamura[105,108,110] showed strong similarity with the L-enzyme-1 reported by Sih.[60] This enzyme has also been described in the literature as α-acetoxy ketone reductase.[110] The E1 (also L-enzyme-1 and YKERI) reported by Nakamura has been the best characterized for substrate specificity[111-114] and although its stereoselectivity matches that of the L-enzyme-2 reported by Sih, it is not clear whether they are identical.

Some of the isolated enzymes have been shown to possess very high stereoselectivities along with broad substrate specificity (Figure 1-17, Table 1-4) however, the need for enzyme isolation and cofactor regeneration has limited the practical utility of these catalysts.

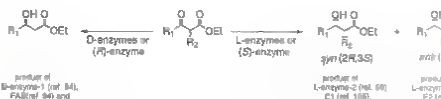


Figure 1-17. Stereoselectivities of β-keto ester reductases isolated from baker's yeast.

Table 1-4 Applications of purified baker s yeast reductases to the synthesis of optically active β hydroxy esters

Enzyme (name is that reported in the original reference)	Substrate (31)			Abs Conf of major product	% yield	% ee	% de	Ref
	R_1	R_2	R_3					
E1[108] (also L-enzyme-1[105] and YKER1[115])	CH_3	H	CH_3	*S*	13	>99		
	CH_3	H	CH_2CH_3	*S*	44	>99		111
	CH_2CH_3	H	CH_2CH_3	*S*	30	>99		
	$ClCH_2$	H	CH_2CH_3	*R*	45	>99		105
	CH_3	CH_3	CH_2CH_3	2*R* 3*S* *syn*		-	>98	108
	CH_3	CH_2CH_3	CH_2CH_3	2*R*,3*S* *syn*	31		>98	111
	CH_3	Allyl	CH_2CH_3	2*R*,3*S* *syn*	70	>98	>98	116
	CH_3	Propargyl	CH_2CH_3	2*R* 3*S* *syn*	72	-	>98	111
E2[108] L-enzyme-2[105] AcKR[110]	$ClCH_2$	H	CH_2CH_3	*R*	84	>99		105
	CH_3	CH_3	CH_2CH_3	2*R* 3*S* *syn*		-	64	108
	CH_3	Allyl	CH_2CH_3	2*S* 3*S* *anti*			88	108
L-enzyme 1[60]	CH_3	CH_3	CH_2CH_3	2*R* 3*S* *syn*	46	98	80	60
	CH_3	Allyl	CH_2CH_3	2*S* 3*S* *anti*	89	98	98	
	CH_3	Propargyl	CH_2CH_3	2*S* 3*S* *anti*	25	98	80	
	CH_3	Benzyl	CH_2CH_3	2*S* 3*S* *anti*	<1	98	62	
L-enzyme 2[60]	CH_3	CH_3	CH_2CH_3	2*R*,3*S* *syn*	26	98	98	60
	CH_3	Allyl	CH_2CH_3	2*R* 3*S* *syn*	27	98	98	
	CH_3	Propargyl	CH_2CH_3	2*R* 3*S* *syn*	45	98	98	
	CH_3	Benzyl	CH_2CH_3	2*R* 3*S* *syn*	4 5	98	80	
2 methyl-3-oxobutyrate reductase[106]	CH_3	CH_3	Benzyl	2*R* 3*S* *syn*	-		77	106

S-enzyme[107]	CH_3	H	CH_2CH_3	*S*		>98		107
	n-propyl	H	CH_2CH_3	*S*		>98		
R-enzyme[107]	CH_3	H	CH_2CH_3	*R*		>98		
	n-propyl	H	CH_2CH_3	*R*		>98		
D-enzyme-1[105]	$ClCH_2$	H	CH_2CH_3	*R*	62	>99		105
D-enzyme 2[105]	$ClCH_2$	H	CH_2CH_3	*R*	80	>99		105
D-enzyme[94]	$ClCH_2$	H	CH_2CH_3	*R*		>97		94
Fatty acid synthetase[94]	$ClCH_2$	H	CH_2CH_3	*R*		>97		94

Improvements in the Stereoselectivity of Baker s Yeast Mediated Reductions of β Keto Esters

The presence of enzymes with different stereoselectivities in baker s yeast has hampered the utility of this biocatalyst for the synthesis of optically pure β hydroxy esters Several ingenious methods have been devised to overcome this problem including changing the substrate structure or concentration [18 99 101 117 118] altering the growing conditions [71 115 120] using organic solvents or a two-phase system [95 96 98] and adding inhibitors for particular enzymes [97 100 121 123]

Substrate modification has proven successful for directing the enantioselectivity of alkyl 4-chloro acetoacetate reductions It was established that the enantioselectivity varied depending on the size of the ester alkoxy group (Table 1-5) and these findings were applied to the synthesis of optically pure L-carnitine [18] Following this report modification of the ester alkoxy group has been shown to affect the stereoselectivity resulting in different degrees of improvement (Table 1-5) [99 101 117 118] Other substrate modifications have included hydrolysis of the ester prior to reduction [124] or substitution at

the α position with a functional group that is easily removed after reduction. The strategy of substrate modification is based on the empirical finding that the reaction involved in the reaction have very different commitment to catalysis for a particular substrate.

Table 1.5 Effect of substrate structure on the stereoselectivity of baker's yeast-mediated reductions of β keto esters

Substrates (30)			% yield	Abs Conf	% ee	h	Ref
R	R_2	R_3					
ClCH	H	CH_2CH_3		*S*	35		18
		$R_3 = n\ C_8H_{17}$		*R*	95		
CH_3	CH_3	CH_2CH_3	75	*syn* 2*R*,3*S*	>98	66	19
		$R_3 = n\ C_8H_{17}$	82	*syn* 2*R*,3*S*	>99	10	
CH_3	Propargyl	CH_2CH_3	66	*syn* 2*R*,3*S*	>98	36	101
		$R_3 = CH_2C(CH_3)_3$	69	*syn* 2*R* 3*S*	>98	54	

Another approach to improving enantioselectivity is based on the kinetic differences between the participating enzymes. Regulated addition of the substrate in control to n certain extent the enantioselectivity towards a given substrate by favoring those enzymes with high affinity for the substrate (Table 1-6). The use of two phase system is likely related to the former strategy since the concentration of the substrate surrounding the cells will also be affected by the partition coefficient of the compound between the two media, as well as the total substrate concentration. In general low substrate concentrations[71] as well as the use of organic solvents favored L

[illegible] 95 98 while [illegible] ab lization [illegible]

[illegible] (Table 1 6) [illegible]

Table 1-6 Effect of substrate concentration on the [illegible] mediated reductions of β keto esters

Substrates (30)			Modification	[illegible]			
R_1	R_2	R_3					
$ClCH_2$	H	CH_2CH_3	none				
			Immobilization in polyurethane (PU)				
			organic solvent toluene		R		
CH_3	H	CH_3	none	23	S		
			organic solvent light petroleum	57	S		
CH_3	H	CH_2CH_3	none	76	S		
			organic solvent light petroleum	69	S	11	
			Immobilization in polyurethane (PU)		S	60	96
			continuous feeding substrate	56	S	95 97	
CH_2CH_2	H	CH_3	none		R		16
			Immobilization in polyurethane (PU)		R	86	16
CH_2CH_2	H	CH_2CH_3	none	61	R	4	97
			organic solvent hexane		S	77	98
nC_5H_{11}	H	CH_2CH_3	none	-	R	86	98
			organic solvent hexane		S	2	98

Some other methodologies have targeted the stereoselectivity for [illegible] substrate by regulating the level of catalytic activity of a particular enzyme The use of inhibitors [illegible]

specific enzyme activities has resulted in good improvements in the stereoselectivity [9, 10, 11]. For example, methyl vinyl ketone and allyl [illegible] the production of the D alcohol [illegible] while ethyl chloroacetate shifted the [illegible] in the opposite direction (Table I.7).[97] Addition of methyl vinyl ketone [illegible] an diastereomer when α substituted β keto esters were tested (Table I.8). [illegible] diastereoselectivity of α alkyl β keto ester reductions was also modified by [illegible] treatment and these results are probably associated with thermal denaturation [illegible] the enzymes (Table I.8).[101, 102]

The addition of inhibitors improves the enantioselectivity but in practice requires a 1:1 ratio of inhibitor:substrate, an increased yeast cell mass (20 [illegible] substrate), and both factors complicate the extraction process and may affect [illegible]. Although Nakamura reported good improvements associated with the use [illegible] later reports suggested much lower stereoselectivity (Table I.7).[11] Addition [illegible] compounds like DMSO and L-cysteine has also been shown to affect the enantioselectivity.[7,123]

[illegible] baker [illegible]-mediated [illegible]

Substrates (30)					
R_1	R	R			
$ClCH_2$	H	$CH\ CH_3$	none		
			allyl alcohol	31	S
				70	R
			ethyl chloro acetate	56	R
CH	H	$CH\ CH_3$	none	76	S
			allyl alcohol	68	S
			ethyl chloro acetate	75	S
$CH\ CH_3$	H	CH	none		R
			allyl alcohol	37	R
			ethyl chloroacetate	51	S
$CH\ CH_3$	H	$CH\ CH_3$	none	61	R
			ethyl chloroacetate	63	S
			ethyl chloroacetate	4	S
n C H	H	CH_2CH_3	none	10	R
			allyl alcohol	6	R
			$(CH_3)_2SO$ glucose	31	R

Table 1 Effect of [illegible] inhibitors [illegible] of β keto ester baker's [illegible]

Substrates (30)			Inhibitor	% yield	Abs Conf			
R	R_2	R_3						
CH	CH_3	CH_2CH_3	none	75	syn 2R 3S	>98	96	99
			Heat (50 °C 30)	69	syn 2R 3S	>98	86	100
			60 mM methyl vinyl ketone (MVK)	72	syn 2R 3S	>98	92	
			Heat + MVK	68	syn 2R 3S	>98	94	
CH_3	CH_2CH_3	CH_2CH_3	none	80	syn 2R 3S	>98	92	100
			Heat (50 °C 30)	72	syn 2R 3S	>98	60	
			60 mM methyl vinyl ketone (MVK)	76	syn 2R 3S	>98	72	
			Heat + MVK	57	syn 2R 3S	>98	54	
CH_3	Allyl	CH_2CH_3	none	71	anti 2S 3S	>98	40	100
			Heat (50 °C 30)	65	syn 2R 3S	>98	0	
			60 mM MVK	66	syn 2R 3S	>98	68	
			Heat + MVK	68	syn 2R 3S	>98	92	
CH_3	Propargyl	CH_2CH_3	None	66	syn 2R 3S	>98	96	101
			Heat (50 °C 30)	79	syn 2R 3S	>98	3	100
			60 mM MVK	73	syn 2R 3S	>98	92	
			Heat + MVK	71	syn 2R 3S	>98	98	

Other methodologies seem to alter the pattern of enzyme product[illegible] the growing conditions. Since several enzymes are involved in these reduct[illegible] vivo concentration of the involved enzymes can be manipulated by modifying the microscopic parameters such as carbon source aeration ging of the culture etc. Related studies establish that starving oxygen limited cultures favored the D hydrox esters while the presence of gluconolactone and active aeration favored the L hydrox esters (Table 1.9).[6]

Table 1.9 Effect of different growing conditions on the enantioselectivity of baker's yeast mediated reductions of β keto esters

Substrates			Modification	% yield	Abs Conf	% ee	Ref
R_1	R	R_2					
$ClCH_2$	H	CH_2CH_3	none		S	55	118
			gluconolactone + active aeration		R	91	120
			oxygen-limited conditions		S	98	
CH_3	H	CH_2CH_3	none	76	S	85	52
			grow 7 days in gluconolactone prior to substrate addition		S	96	119
			starving for 4 days in 5% ethanol prior to substrate addition	70	S	94	
			gluconolactone + active aeration		S	>98	120
CH_2CH_3	H	CH_2CH_3	none	61	R	4	97
			gluconolactone + active aeration		S	81	120
			oxygen limited conditions		R	96	

Use of Genetic Engineering to Improve the Enantioselectivity of Yeast Carbonyl Reductions – An Overview of this Work

The presence of several reductases with overlapping substrate specificities and stereoselectivities explains the mixture of alcohol products produced in baker's yeast mediated reductions of β keto esters. While purified yeast β keto reductases provide highly stereoselective biocatalysts, their utility is hampered by the need for enzyme isolation and cofactor regeneration. On the other hand, while baker's yeast provides a very handy inexpensive reagent but also shows modest stereoselectivity for reductions of many substrates. The optimal solution to the approach to β hydroxy esters would be one that combines the advantages of isolated enzymes with those of the whole cell system.

Most of the reported strategies for improving the enantioselectivity of yeast esters baker's yeast mediated reductions are aimed at decreasing the catalytic activity of competing enzymes while leaving that of the target enzyme largely undisturbed. The major difficulty with all of these approaches is that they are empirical and the results of the reductions of novel substrates are not easily predictable. On the other hand, recombinant DNA technology provides a potentially powerful route to improving the stereoselectivities of yeast reductions in a rational way. The goal of this project is to engineer yeast strains that control the stereoselectivities of the individual enzymes.

Two basic genetic tools allow us to manipulate the enzyme expression in yeast and thus the relative concentration of the catalysts: gene overexpression and knockout. Expression systems for the enzyme of interest can be constructed

large amount of that enzyme will be produced (Figure 1.18). The [illegible] catalyst concentrations should affect the stereoselectivity of the new [illegible] competing enzyme that lowers the stereoselectivity of a biotransformation. [illegible] corresponding gene can be knocked out through homologous recombination [illegible] wild type gene is replaced with a non-functional variant constructed *in vitro*. [illegible] The absence of the competing enzyme in the mutant strain will result in [illegible] stereoselectivity.

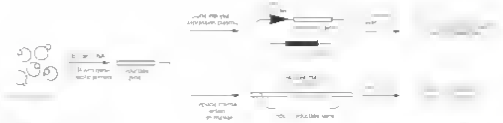


Figure 1.18. Overview of the general strategy for the genetic design of [illegible] yeast strains.

Three enzymes (fatty acid synthetase and two L-enzymes) had been [illegible] the major participants in the reduction of β-keto esters by baker's yeast. Fatty [illegible] synthetase (FAS) catalyzes the reductions of β-keto esters to yield the D-[illegible] the L-enzymes result in formation of the L-carbinols (Figure 1.19).[81,82] The L-[illegible] also accept α-substituted β-keto esters as substrates and they yield opposite diastereomers.[80] The L-enzyme responsible for production of the syn diastereomer [illegible] classified as a member of the aldo-keto reductase family, and will be referred [illegible] keto reductase throughout this work. The second L-enzyme was identified [illegible]

ketone reductase and this name will be used throughout this thesis. The previous nomenclature

Figure 1.19 Three enzymes have been reported as the major β keto ester reductases present in baker's yeast

The genetic design approach required the identification of the genes encoding each enzyme. Fatty acid synthetase is encoded by the *FAS1* and *FAS2* genes [refs 1] which have been previously cloned.[127] Aldo keto reductase has been purified [illegible] its N-terminal amino acid sequence was determined [109] based on this data the gene encoding for this enzyme was identified as *YPR1*. Amino acid data was also reported for α acetoxy ketone reductase and was in complete agreement with an hypothetical protein encoded by the open reading frame ygr105w.[128] However the results presented in this work indicate that this assignment was incorrect. To identify the correct gene the α acetoxy ketone reductase was purified and internal Lys C fragments were sequenced. The data obtained corresponded precisely to the protein encoded by the *GRE2* gene.

Strains over-expressing each of these three reductases were constructed and analyzed for changes in the enantio and diastereoselectivity of β keto ester reduction. Expression systems based on the *S. cerevisiae* expression vector pYES2 were constructed

[illegible]-ketoreductase and fatty acid synthetase wh[illegible]

acetoxy ketone reductase was based on pAAH5 [129]

Strains in which each of these reductases were knocked out were analy[illegible] their effects on the stereoselectivities of β keto ester reductions. The strain of [illegible] knockout of the *YPR1* gene was created by micro homology gene replacement. [illegible] *fas2* mutant strains have been used along this work. The first mutant was p[illegible] Schweizer and obtained from a strain bank (ATCC 264031 [131]) and a second [illegible] carrying the complete deletion of the gene (Δ*fas2*) was obtained by sporulation [illegible] correspondiog heterozygous diploid. The strain carrying the knock out of the *OYE2* gene was obtained from the Euroscarf consortium.

The construction of these recombinant strains as well as the performance of the different designed yeast on the reduction of several β keto esters are presented in Chapter 3. Although the results obtained from this first generation of engineered s. [illegible] provided good improvements on the stereoselectivity of baker's yeast reductases, incomplete stereoselectivity was still a common outcome of these biotransformations. These results represented new challenges since it indicated that more reductases than those originally thought were involved in the reduction and implied that a combination of knock out and over expression strategies might be necessary to achieve complete stereoselectivity.

In order to achieve higher levels of diastereo- and enantioselectivities, [illegible] combining overexpression and knockout strategies were constructed. A [illegible] diastereoselective strain was created by over-expressing aldo keto reductase in the strain carrying the deletion of the α acetoxy ketone reductase. Similarly, a designed yeast [illegible]

the opposite stereoselectivity was obtained by combining the [illegible]
ui with the α-acetoxy ketone reductase. A double deletion mutant [illegible]
n both *S* enantioselective enzymes served as background for the expressio[illegible]
yielding an *R*-enantioselective strain. Finally, over-expression of α-acetoxy
reductase in the *Δgre2* deletion mutant resulted in an *S*-enantioselective [illegible]
The construction of this second generation of "designed yeast" alone with [illegible]
improvements in the stereoselectivity of β keto ester reductions are presen[illegible]
4

Since the outcome of the experiments with single mutants presented evidence that more reductases were involved in β keto ester reductions, identify these reductases were undertaken. Based on sequence homol[illegible]
reductases, several candidates were identified [11]. Three yeast open reading presented strong homology with α-acetoxy ketone reductase, although no information was available about these hypothetical proteins. In a similar s[illegible]
homologs of the aldo-keto reductase were identified. Three of these enzymes Gre3p and Ara1p) had been previously isolated and characterized as reduc[illegible]
although their potential role on β keto ester reductions was never addressed. kinship with aldo-keto reductase, and to learn whether these enzymes might be stereoselective reductions of β keto esters, engineered yeast and *E. coli* strains each of them were created. The recombinant strains were tested for reduction β keto esters along with some other carbonyl compounds. The results presented

Chapter 3 indicate that Gcy1p and Gre3p are indeed β ket

Ara1p is involved in α keto ester reductions

In summary, yeast strains with high stereoselectivity were obtain

ck- ot and over-expression of the three enzymes that were previously

sponsible of β keto ester reductions in baker's yeast. These re

ential of the designed yeast approach to tailor the stereospecificity

provide useful biocatalysts for these transformations. In addition, the

long this work uncovered the presence of more reductases involved in

reductions of β keto esters. Some of these other enzymes have been identi

ir homology with known reductases and their expression in *E. coli*. Th

probably be useful for the identification of the remaining reductases in bake

improve the rational design of yeast strains with a particular stereoselect

CHAPTER 2
IDENTIFICATION OF THE GENES ENCODING FATTY ACID SYNTHETASE AND ALDO KETO REDUCTASE ISOLATION AND CHARACTERIZATION OF α-ACETOXY KETONE REDUCTASE

The genetic design approach to improving the stereoselectivities of yeast reductions requires the identification of the genes encoding each relevant enzyme. The availability of the complete genome sequence of baker's yeast [36] along with the identification of some of the enzymes involved in these reductions [94,105] provided in two cases the identities of the corresponding genes. By contrast, it was necessary to isolate the third major β-keto ester reductase to obtain the required information.

Fatty Acid Synthetase

Several groups identified fatty acid synthetase (FAS) as the major enzyme involved in the reduction of β-keto esters to yield D-carbinols [94,105,107]. Yeast fatty acid synthetase is a multifunctional enzyme composed of two non-identical subunits, α and β which are organized in an $\alpha_6\beta_6$ dodecameric complex. These subunits are encoded by the *FAS2* and *FAS1* genes, respectively. Experiments with a mutant *fas2* deficient strain (ATCC 26403) have shown that the 3-oxoacyl reductase component of the FAS was responsible for the reductase activity [94]. Conflicting reports have appeared in the literature concerning whether the complete FAS complex was responsible for the reductase activity observed in whole cells or a single subunit is sufficient. The three groups that assigned a β-keto ester reductase activity to FAS reported different molecular

weights. While one group reported a MW of 2400 kDa[104] that can be assigned to the $\alpha_6\beta_6$ complex, the two other groups reported values of 1600[105] ($\alpha_6\beta_6$) and 800 kDa[107] (β) respectively. Studies by Heidlas et al. presented evidence that the β keto ester reductase activity was present in the $\alpha_6\beta_6$ subunit but the $\alpha_6\beta_6$ complex showed no capacity to catalyze these reductions.[107]

Aldo Keto Reductase

Two L-enzymes of similar molecular weight (~ 32 kDa) that use NADPH as the sole cofactor and have very similar kinetic parameters have been isolated by two groups and are likely to be the same aldo keto reductase.[60,109,110] The N-terminal amino acid sequence of this protein was reported by Nakamura as P-A T L K N S S A T L K L S T G.[109] a BLAST search using the *S. cerevisiae* genome database indicated complete match with Ypr1p, an enzyme that belongs to the aldo keto reductase superfamily encoded by the *YPR1* gene. Later reports in the literature suggested that this enzyme activity might also be due to aldose reductase I (YHR104w), aldose reductase II (YDL124w), or aldehyde reductase I (YBR149w).[103] Although all these latter enzymes belong to the same family, the only protein whose sequence completely matched the reported data was Ypr1p.

α Acetoxy Ketone Reductase

L-enzyme-2 was isolated from oriental baker's yeast using ethyl 4-chloro acetoacetate[109] as substrate, while 1-acetoxy-2 propanone was used for the isolation of α acetoxy ketone reductase from the same organism.[118] Protein analysis later revealed that

[illegible] α acetoxy ketone reductase [illegible] The [illegible] reductase was estimated to be 36 kDa by SDS PAGE and 39 kDa by gel [illegible] This indicated that the enzyme was composed of a single polypeptide chain [illegible] enzyme also seems very similar to a protein isolated by Sih from Red St[illegible] though this was a dimer of 74 kDa [60] Whether this difference is strain related or [illegible] the two distinct is not clear however the similar substrate specificity and stereo[illegible] issues that these might be the same enzyme

The N-terminal amino acid sequence of α acetoxy ketone reductase was [illegible] to be T A P L V V L-G N-P L L D F Q A[109-110] and a BLAST search using the *Saccharomyces cerevisiae* genome database indicated that this enzyme matched a 36.4 kDa [illegible] protein encoded by the YJR105w open reading frame (ORF) and this [illegible] later reported by Nakamura in the literature [61] However as will become clear in the following sections this assignment was incorrect To identify the correct [illegible] α acetoxy ketone reductase was purified according to the procedure reported by Sih and the amino acid sequence of internal Lys-C fragments was determined [illegible] from three separate fragments corresponded precisely to the protein encoded by the *Saccharomyces cerevisiae GRE2* gene which has sequence similarity to plant dihydroflavonol and cinnamoyl CoA reductases

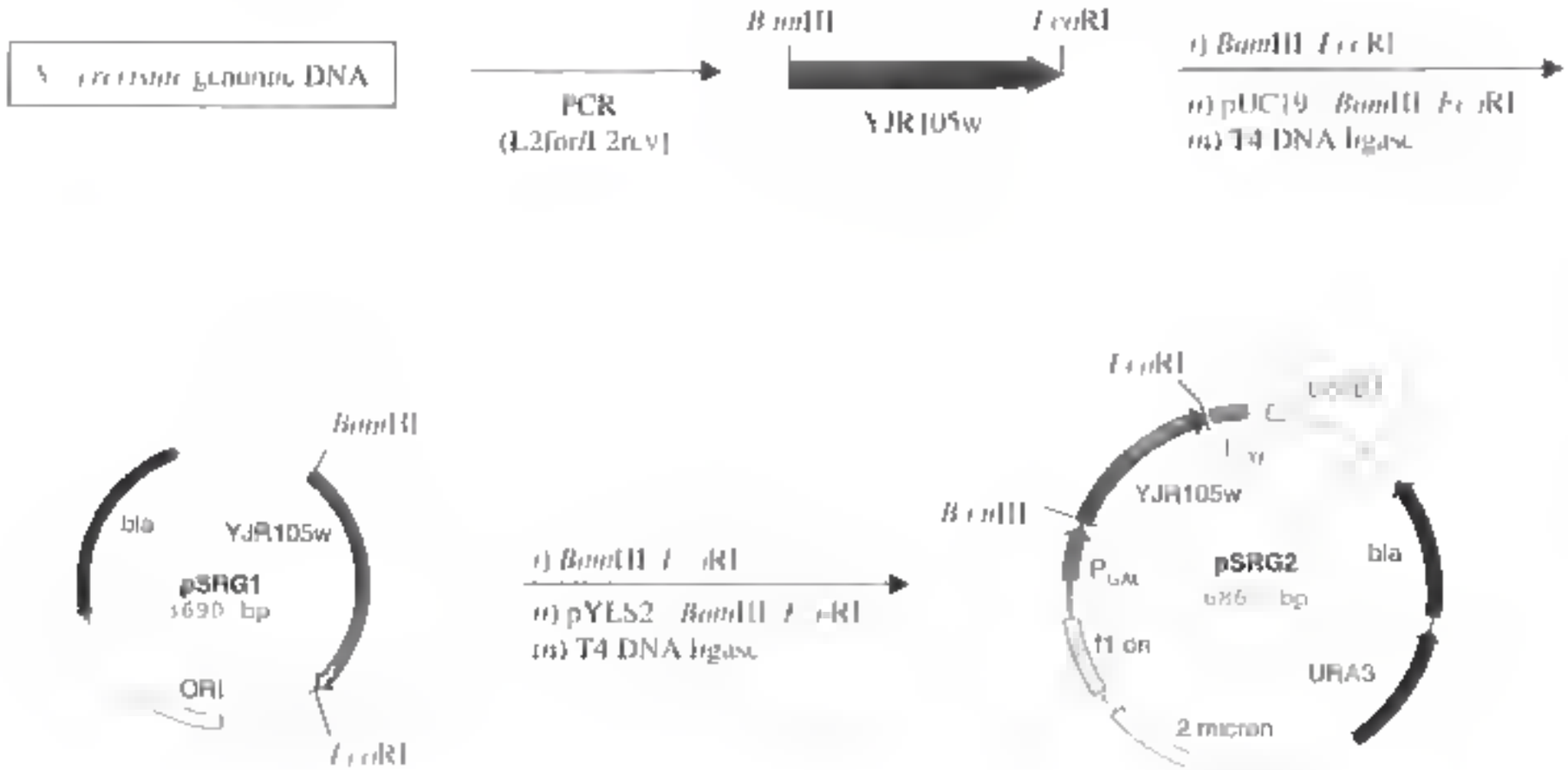
<u>YJR105w Does Not Encode α Acetoxy Ketone Reductase</u>

The first indication that the protein encoded by YJR105w was not [illegible] ketone ester reductase came from sequence similarity (BLAST) searches These revealed [illegible] relationship between the YJR105w ORF (open reading frame) and adenylate kinase [illegible]

YJR105w ORF actually corresponded to the [illegible] strains carrying either a deletion or an over expression system for the YJR105w ORF were created. As was suspected neither deletion nor over expression of the YJR105w ORF had any effect on the outcomes of whole cell mediated β keto ester reductions. Moreover analysis of the soluble protein extract from the deletion mutant indicated that the α acetoxy ketone reductase activity was still present in the mutant strain, thus confirming the notion that the N terminal amino acid sequence for α-acetoxy ketone reductase had been misreported. Details of these studies are given below.

Over Expression of the Protein Encoded by YJR105w

An over expression system for the enzyme encoded by the YJR105w ORF was based on the yeast expression vector pYES2.[38] The strategy used to create this plasmid is summarized in Figure 2.1. Appropriate restriction sites for the cloning strategy were incorporated in the PCR primers JL2for, JL2rev1 and the complete YJR105w ORF was PCR amplified from *S. cerevisiae* genomic DNA and then cloned into pUC19 after digestion with *Bam*HI and *Eco*RI. Subcloning of the *Eco*RI – *Bam*HI cassette into these sites of pYES2 resulted in the expression system pSRG2. In this vector protein expression is controlled by the GAL promoter and is induced by a change in carbon source. The 2μ origin of replication maintains the plasmid copy number at 140 per yeast cell and the presence of the URA3 gene allows selection of yeast transformants in uracil depleted media. This plasmid also contains the ampicillin resistance gene (*bla*) and the *colE1* origin of replication for maintenance and cloning in *E. coli*.


genomic DNA
PCR
(L2forl 2rev)
BamHI
EcoRI
YJR105w
i) BamHI EcoRI
ii) pUC19 BamHI EcoRI
iii) T4 DNA ligase
BamHI
bla
YJR105w
pSRG1
5690 bp
ORI
EcoRI
i) BamHI EcoRI
ii) pYES2 BamHI EcoRI
iii) T4 DNA ligase
EcoRI
YJR105w
BamHI
pSRG2
bla
f1 ori
URA3
2 micron

Transformation of the yeast strains 15C and InvSc1 with pSRG2 [illegible] recombinant strains that potentially over-expressed the protein encoded by YJR105w ORF 15C[pSRG2) and InvSc1(pSRG2) Both strains were tested for changes in the stereoselectivity of β keto ester reductions It was expected that if the enzyme [illegible] expressed were α acetoxy ketone reductase this would effect the stereochemistry resulting in the production of more L product and more *anti* diastereomer in the unmodified strains However the results obtained with two test substrates [illegible] substrates for α acetoxy ketone reductase showed no detectable difference between the recombinant and parental strains (*vide infra* Table 2 3) strongly suggesting that the protein corresponding to the YJR105w ORF is not a β keto ester reductase

Construction of a Mutant Strain Carrying the Deletion of the YJR105w

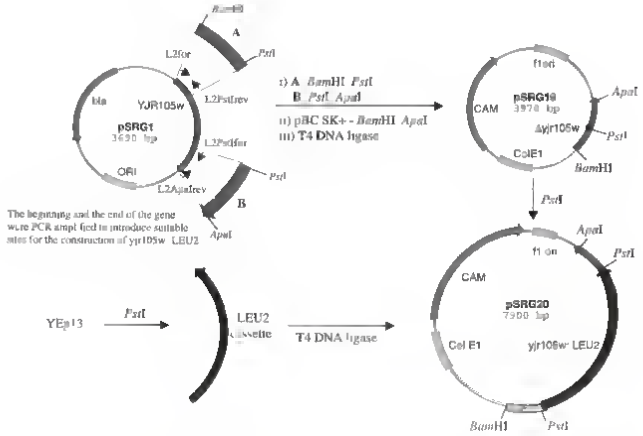
The results obtained from over-expressing the YJR105w ORF prompted the design construction and testing of a strain carrying a knock out of YJR105w [illegible] the notion that this protein was not a β-keto ester reductase

A mutated yjr105w allele was generated *in vitro* by replacing a 400 bp internal region in the ORF with a marker LEU2 cassette using a leucine auxotroph strain (Figure 2 2) In a first step the beginning and the end of the YJR105w ORF were PCR amplified with primers that introduced suitable restriction sites for insertion of the LEU 2 cassette as well as for the release of the final construct The PCR products were [illegible] with the appropriate restriction enzymes and cloned simultaneously in pBC SK+ [illegible] pSRG19 This plasmid carried a partial deletion allele of the YJR105w with a [illegible] linking both extremes of the gene This site was used to introduce the LEU 2 cassette [illegible] YEp13 yielding the final construct (pSRG20) in which the internal part of YJR105w ORF

was substituted by the LEU2 marker. The yjl105w::LEU2 deletion cassette was isolated from pSRG20 as an *Apa*I – *Bam*HI fragment and used directly for transformation of *Saccharomyces cerevisiae* 15C. The homologous regions of this transforming linear DNA fragment guaranteed high recombination efficiency. The strains in which replacement of the chromosomal YJR105w locus by the deletion mutant have occurred were selected on leucine depleted media. This process resulted in several colonies from which the strain JS2 was further characterized.

The mutant strain JS2 carrying the yjl105w::LEU2 deletion was characterized for both the presence of the YJR105w knock out and absence of the wild type allele by PCR and southern blot techniques. Genomic DNA was isolated from JS2 and from the parental strain 15C. The plasmid pSRG20 containing the yjl105w::LEU2 construct was used as a positive control in the PCR experiments.

Figure 2 2 Construction of the yjr105w LEU2 dsDNA used to generate JS2 (yeast strain carrying the YJR105w deletion)



The beginning and the end of the gene were PCR ampl fied to introduce suitable sites for the construction of yjr105w::LEU2.

PCR characterization of the JS2 mutant strain

A first set of primers (L2seq3f/ L2rev) was intended to amplify [illegible] YJR105w while the second set (LeucineFor/ L2rev) was designed [illegible] replacement of the wild type allele by yjr105w::LEU2 (Figure 2.3

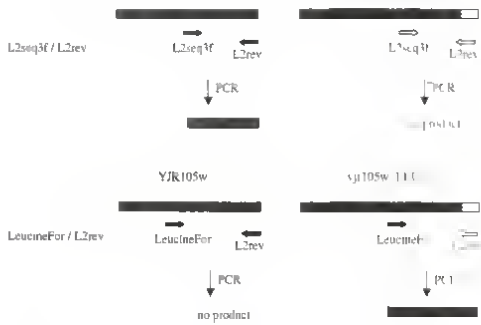


Figure 2.3 Characterization of 15C(yjr105w::LEU2) based on PCR experiments

The results obtained from the PCR experiments with the designed primers are summarized in Table 2.1. The first set of primers amplified a 500 bp fragment of the YJR105w when the parental strain (15C) DNA was used as template. substitution of the PCR template by JS2 genomic DNA resulted in no product formation, indicating that the

YJR105w wild type gene was no longer present in JS2. The yjr105w::LEU2 [illegible] pSRG20 was used as control template in the experiment with the second set of primers resulting in formation of a 1000 bp product. The same result was obtained with JS2 but not with 15C confirming the presence of the yjr105w::LEU2 insertion in the JS2 genome. An additional confirmation was obtained by using the set of primers originally designed to PCR amplify the YJR105w ORF (L2for L2rev). As expected a 1000 bp product was seen when using 15C DNA as template, however substitution of the template by either pSRG20 or JS2 yielded no PCR product since the predicted 4500 bp product was too long to be amplified under the experimental conditions. The fact that JS2 presented the same results as pSRG20 further confirmed that the gene replacement was successful and that the wild type allele is absent from this strain.

Table 2.1. Characterization of 15C(yjr105w::LEU2) based on PCR experiments.

Primer set		Template for the PCR experiment		
		15C	pSRG20	JS2
L2seq3f	L2rev	500 bp	no product observed	no product observed
LeucineFor	L2rev	no product observed	1000 bp	1000 bp
L2for	L2rev	1000 bp	no product observed	no product observed

Southern blot characterization of the JS2 mutant strain

Two Southern blot probes were employed to verify the gene disruption of the YJR105w ORF. Probe 1 was designed to bind both the wild type allele and the mutated gene although it would indicate differences in the restriction site patterns depending on the nature of the YJR105w locus. The probe was constructed by PCR amplification of the extreme of the YJR105w gene that is still present in yjr105w::LEU2 (Figure 2.4). The second probe targeted the internal fragment of YJR105w that was replaced by LEU2 in JS2. This probe was designed to indicate the presence or absence of the wild type allele (Figure 2.4). The PCR primer L2seq3f (26bp) was used as probe for this second experiment.

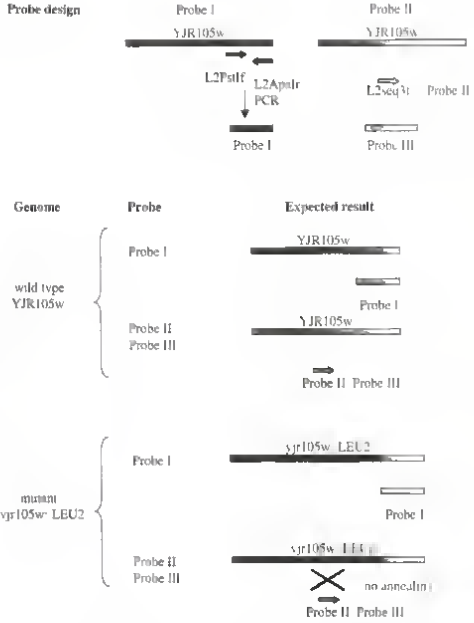
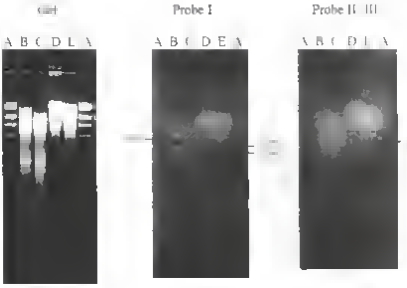


Figure 2-4 Design of the southern blot probes and characterization of the mutant JS2.

Two enzymes *EcoR*I and *Sac*I that resulted in different restriction patterns for the mutant and the parental strain were used for the digestion of 15C and JS2 genomic DNA

The digested DNAs were run side by side in an agarose gel blotted [illegible] membrane and hybridized with the probes The results obtained are presented [illegible] 5 and summarized in Table 2 2 As expected probe I hybridized to both the mutant JS1 and the wild type (15C) allele. The difference in size in between the hybridized DNA fragments agreed with those predicted, confirming the replacement of the YJR105w allele by the mutated yjr105w::LEU2 The *Eco*RI digested DNA did not hybridize with the second probe both for the mutant and the wild type DNA One possibility is that the designed probe might be too short to form stable duplexes, another probe targeting the same region was therefore constructed (Probe III) The *Kpn*I – *Xcm*I fragment of the cloned YJR105w in pSRG1 yielded a 360 bp probe for this region Repeating the Southern blots using Probe III still showed no binding to genomic DNA from either strain digested with *Eco*RI The origin of these results is not clear

The absence of the YJR105w internal segment in the JS2 mutant was verified with the results obtained with the second probe and *Sac*I digested genomic DNA As predicted this probe annealed only to the wild type (15C) DNA and not to JS2 indicating the lack of the wild type allele in the mutant strain (Figure 2 5)



A MW marker E unbdn DNA *Hin*DIII digested
B JS2 DNA - *Eco*RI digested D JS2 DNA *Sac*I digested
C 15C DNA *Eco*RI digested E - 15C DNA - *Sac*I digested

Figure 2.5 Southern blot hybridization experiments for characterization of the yjl105w LEU2 knock out in JS2

Table 2.2 Results from the southern blot characterization of the JS2 mutant strain. Comparison of expected and actual results for the wild type (15C) and the mutant JS2 strains

Probe	Digestion enzyme	Expected results (size bp)		Actual results (size bp)	
		15C	JS2	15C	JS2
Probe I	*Eco*RI	6884	4619	6900	4600
	*Sac*I	3501	3300	3500	3300
Probe II	*Eco*RI	6884			
	*Sac*I	3501		3500	

Biotransformations with the Engineered Strains

Two ketones were selected to study the effect of over-expressing or knocking out the YJR105w ORF (Figure 2-6). Both ketones had been shown to be substrates for purified α acetoxy ketone reductase [103]. The low enantioselectivity associated with the reduction of ethyl 3 oxo pentanoate by unmodified baker's yeast provided a useful substrate for testing any modification in the expression pattern of reductases. Analysis of the effects on the reduction of ethyl α allyl acetoacetate should also reflect those changes since the acetoxy ketone reductase produces the *anti* diastereomer whereas the aldo keto reductase affords the *syn* alcohol.

32

a R_1 = Et, R_2 = H, R_3 = Et
b R_1 = Me, R_2 = allyl, R_3 = Et

Figure 2-6. Model substrates used for testing the effect of YJR105w over-expression and knock out on the stereoselectivity of whole cell yeast mediated β keto ester reductions.

The engineered strains carrying the over-expression and the knock out of the YJR105w ORF were analyzed for changes in stereoselectivity of whole cell mediated reduction with both test compounds. The over expression of α acetoxy ketone reductase should increase production of the (*S*)-alcohol from **32a** and favor the *anti* product from **32b** relative to the unmodified host. However, this was not observed, and the stereoselectivity of the over-expression strain was virtually identical to that of the control

train (Table 2-3). Moreover, deletion of the YJR105w ORF also h[illegible] reductions of the model ketones. These results are consistent with [illegible] YJR105w has been mis reported as encoding a reductase.

Table 2.3. Effects of YJR105w over expression 115C(pSRG2)) and knock-[illegible] the stereoselectivity of whole cell yeast mediated β keto ester reductions.

Substrate (32)			over-expression	control	knock-out
R_1	R_2	R_3			
Et	H	Et	70 % ee (R)	70 % ee (R)	70 % ee (R)
Me	allyl	Et	6 % de (syn)	17 % de (syn)	14 % de (syn)

The α Acetoxy Ketone Reductase Activity Was Still Present in JS2

The purification of α acetoxy ketone reductase along with other yeast reductases has been previously reported in the literature, and it has been shown that anion exchange chromatography using a DEAE column clearly separated the three major ethyl 4-chloro-acetoacetate reductase activities.[105] In order to test whether the α-acetoxy ketone reductase activity was still present in the knock out strain JS2, the elution profile of a crude extract from the mutant strain applied onto a DEAE column was analyzed and compared to that of the parental strain and the data reported by Nakamura.[105] These results provided conclusive evidence that the α acetoxy ketone reductase activity was still present in a strain in which the YJR105w ORF was mutated, confirming the notion that YJR105w does not encode this activity.

The mutant and parental strains, JS2 and 15C, were grown in large scale to obtain a final mass of 120-130 g of wet cells. The cells were lysed and the soluble protein

extract was separated from membrane fractions by centrifugation. Once [illegible] concentrated to 40 mL, the crude extracts were applied to a DEAE sephacel c[illegible] 2.5 cm) equilibrated with 10 mM phosphate buffer pH 7. After washing the column with 50 mL of this buffer, the bound proteins were eluted with a linear gradient consisting of KCl (0 – 0.4 M) in a total volume of 2 L of the equilibration buffer. The presence of β keto ester reductase activity was followed by spectrophotometric assays using ethyl 4 chloro-acetoacetate as substrate. Using extracts prepared from both strains, two major peaks with β keto ester reductase activity were eluted at 0.02 and 0.14 M KCl. Analysis of β-keto ester reductions catalyzed by both fractions revealed the presence of an L-enzyme with the characteristics of aldo keto reductase in the first peak and those of α-acetoxy ketone reductase in the second peak. The enzyme fractions were incubated with the substrate, NADPH and a regeneration system composed of glucose-6 phosphate and glucose-6-phosphate dehydrogenase. After 48 h the reaction was extracted and the enantio- or diastereomeric excess was determined by chiral phase GC (Table 2.4). The first peak catalyzed the reduction of both substrates to yield either ethyl 3-(*S*)-hydroxy butanoate or ethyl 2-(*R*)-allyl 3-(*S*) hydroxy butanoate as the sole products. These results are in agreement with the presence of aldo keto reductase (Ypr1p) in this fraction [8,9,10]. The results obtained with the second fraction indicated that a mixture of enzymes was present, despite this, both strains exhibited the same pattern and the major activity present corresponded to α-acetoxy ketone reductase. Reduction of ethyl acetoacetate with the second enzyme fraction resulted in production of ethyl 3-(*S*) hydroxy butanoate as the major product (83 % ee), the incomplete enantioselectivity probably arise from co-elution of the D-enzyme, a minor β keto ester reductase that has been previously reported [8,

The incomplete diastereoselectivity in the reduction of **32b** indicates that [illegible] ester reductase with similar activity to aldo keto reductase was co-eluting with α-acetoxy ketone reductase. This was a repeated observation during the isolation of α-acetoxy ketone reductase and a clue that more reductases than those previously reported in the literature were present in baker's yeast.

Table 2-4 Stereoselectivity of the two major reductase fractions isolated from the mutant strain JS2 and the parental strain 15C

Substrate (32)			15C (control)		JS2 (mutant)	
R_1	R_2	R_3	Peak 1	Peak 2	Peak 1	Peak 2
Me	H	Et	> 98 % ee (*S*)	83 % ee (*S*)	> 98 % ee (*S*)	[illegible] ee (*S*)
Me	allyl	Et	> 98 % de (2*R*,3*S*)	45 % de (2*R*,3*S*)	> 98 % de (2*R*,3*S*)	45 % de (2*R*,3*S*)

Identification of the Gene Encoding α-Acetoxy Ketone Reductase

The identification of the gene encoding α-acetoxy ketone reductase was critical to the success of this project since this enzyme is a major player in the reduction of β keto esters by whole cells of baker's yeast. The enzyme was therefore purified according to Shieh and its amino acid sequence was determined unequivocally show that it is the product of the *GRE2* gene.

Purification of the α-Acetoxy Ketone Reductase

α-Acetoxy ketone reductase was purified 300-fold following the procedure described by Shieh[137] with minor modifications. The dry yeast cells were washed with water twice, suspended in cold lysis buffer and disrupted in a French pressure cell. The residual cells and membrane components were removed by centrifugation and the mitochondrial fraction was separated by ultracentrifugation. Protein fractions were precipitated out from the clear cell extract by addition of $(NH_4)_2SO_4$. The 80 - 90 % saturation fractions containing all the reductase activity were dialyzed to eliminate excess salt, then the protein was purified using several chromatography steps, including two affinity columns. The procedure is summarized in the purification chart (Table 2.5) and a description of each step is given below.

Anion exchange chromatography

The dialyzate from the 50 – 80 % $(NH_4)_2SO_4$ saturation fraction was applied to a DEAE-sephacel column (5.4×2.5 cm) equilibrated with 10mM Tris-HCl (pH 7.5). The column was eluted with 150 mL of this buffer followed by a linear gradient of NaCl (0 – 0.5 M) in a total volume of 1L of the original buffer. When the elution was completed the column was further washed with the same buffer containing 1M NaCl. The proteins were eluted at an average flow rate of 2.5 mL/h and fractions of 8 mL were collected. The matrix used for this separation (DEAE-sephacel) shrank during the elution resulting in variable flow rates and variable fraction sizes. This problem could likely have been avoided if DEAE-sepharose had been used, as reported in the original procedure.

Three major peaks with reductase activity were eluted from this column. The first peak eluted before starting the gradient and it was assigned to the aldo keto reductase encoded by the *YPR1* gene. The two other peaks eluted between 0.2 and 0.3 M NaCl and their peaks of activity overlapped. In order to avoid losing enzyme, the two peaks (fractions 26-51) were pooled together and concentrated by ultrafiltration. The sample was then dialyzed overnight against 4 L of 5mM potassium phosphate, pH 7.5.

Hydroxyapatite chromatography

The dialysate from the previous step was loaded onto an Hypatite C column (2.1×1.5 cm) previously equilibrated with 5mM potassium phosphate, pH 7.5. A linear gradient of potassium phosphate 5 mM – 150 mM, pH 7.5, total volume 500 mL, was used to elute the proteins at a flow rate of 25 mL/h. Fractions of 8 mL were collected. Fractions with reductase activity were eluted from nearly the beginning of the gradient almost up to the end. This was probably due to the column being overloaded by the amount of sample, although the reported procedure presented a similar behavior. As expected, this step barely contributed to the overall purification, however, it may have been useful for removing some protein that would be difficult to remove by other means. Despite the low improvement in the purification obtained in this step, the purification was continued according to the protocol. Fractions 12-64 were pooled and concentrated by ultrafiltration. The buffer was changed by overnight dialysis against 2 L of 20mM Tris-HCl, 50mM NaCl, pH 7.5.

Gel filtration chromatography

A sephacryl S-200 column (85×2.5 cm) previously equilibrated with 20mM Tris-HCl, 50mM NaCl, pH 7.5 was loaded with the sample obtained after Hypatite C

chromatography The proteins were eluted with 500 mL of the same buffer at a flow rate of 20 mL/h and fractions of 10 mL were collected A big envelope of protein elution was seen between fractions 14 to 30 and two major peaks with reductase activity were observed The first peak eluted between fractions 15 18 and the second major peak eluted between fractions 20 and 25 These two peaks were primarily assigned as being the D-enzyme and the α acetoxy ketone reductase respectively The second peak was concentrated by ultrafiltration and dialyzed against 10 mM Tris HCl pH 7.5 overnight

Matrex Blue A chromatography

The sample from the previous step was loaded onto a Matrex Blue A column (12×1 5 cm) equilibrated with 10 mM Tris-HCl pH 7.5 and the column was closed for 30 minutes to favor interaction between the dye and the proteins The column was then washed with 100 mL of the equilibration buffer in order to elute weakly binding proteins A linear gradient of NaCl 0-0 5 M) in 300 mL of the same buffer was used to elute the column The flow rate was set at 23 mL/h and fractions of 8 mL were collected The reductase peak eluted at 0 25 NaCl Fractions 32-42 which showed reductase activity were pooled together and concentrated by ultrafiltration The sample was then dialyzed to change the buffer to 20 mM sodium phosphate pH 7 5

Matrex Red A chromatography

A Matrex Red A column (12×1 5 cm) equilibrated with 20 mM sodium phosphate (pH 7 5) was loaded with the dialyzate from the previous step The column was closed for 30 minutes before elution, then washed with 190 mL of the equilibration buffer Next elution of the protein was performed with a linear gradient of NaCl 0-0.5 M in the same

buffer. Starting in fraction 10 the enzyme of interest eluted all alone until fraction 44. Fractions 10-32 also showed presence of the red dye leaching out of the column explaining the early elution of the enzyme. Fractions 33-46 eluted at the expected NaCl concentration and no dye was present on these fractions. The two sets of fractions were concentrated separately and dialyzed first against 20 mM sodium phosphate containing 0.5 M NaCl in order to break the enzyme-dye interaction, then against 20 mM sodium phosphate. Both fractions were further purified by a second Matrex Red A chromatography step.

For each fraction, the column was equilibrated, loaded and eluted as described in the previous paragraph. The flow rate was set at 12 mL/h and fractions of 8 mL were collected. Both samples from the initial Matrex Red A chromatography exhibited reductase activity in a protein peak eluting at 0.2 M NaCl. The peak eluted from the second column gave higher enzyme activity and it was used for the characterization of this enzyme. SDS polyacrylamide gel electrophoresis of fractions 28-42 indicated the presence of only one protein band of 38 kDa; however when the fractions were further concentrated the presence of two other minor bands was detected. A correlation between fractions with activity and presence or absence of the different bands established that the major band was the only one present in all the fractions showing reductase activity and that its concentration varied according to the activity. Fractions 35-36 were pooled, concentrated and used for amino acid sequencing. Fractions 28-34 and 37-42 were pooled and concentrated; then this sample was used to characterize the enantio- and diastereoselectivity of the isolated enzyme.

A summary of the purification of this reductase is shown in Table 2.5. The procedure resulted in a 300 fold purification of the enzyme with a 2% overall yield in 73 mg. The MW of 38 kDa is in agreement with previous reports [19,110,137] and the specific activity for ethyl 4-chloro aceto acetate is of the same order of magnitude as that reported by Shieh [137]. From the purification factors obtained in the different steps it is clear that the major contributions to the purification were provided by the anion exchange and the affinity columns. However it is important to note that the gel filtration step separated at least two reductases still present in the preparation and this explains the lower specific activity seen after this step. Although the contribution of this step is not apparent from the purification chart it provided the means for separating the enzyme of interest from other reductases.

Table 2.5 Summary of the purification of [illegible] kinase isolated from baker's yeast

Fraction	Volume (ml)	Protein (mg)	Total Act ($\times 10^{[illegible]}$ units)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude Extract	210	11130	1422	128		100
$(NH_4)_2SO_4$ 50-80 % fraction	120	3084	511	166	1.3	36
DEAE Sephacel	55	438	266	608	4.7	19
Hypatite C	50	409	263	642	5	18
Sephacryl S-200	15	177	98	554	4.3	
Matrex Blue A	16	115	143	1245	9.7	[illegible]
Matrex Red A	15	0.73	27.5	37671	295	[illegible]

Stereoselectivity and Substrate Specificity of the Isolated Enzyme

The substrate specificity and the stereoselectivity of the isolated α acetoxy ketone reductase were assayed with several substrates (Table 2-6). The enzyme was shown to reduce acetoacetol to 1-acetoxy 2 propanol, suggesting that the enzyme was indeed an α acetoxy ketone reductase. Only one enzyme from baker's yeast capable of reducing this substrate has been reported.[118] This group also showed that the same enzyme also possessed the capability of reducing β keto esters to yield the 3*S* enantiomer and the *anti* diastereomer.[109,110] The results presented here strongly argue that the isolated enzyme is the same as that isolated by Nakamura.

Several ketones were used to probe the behavior of the purified enzyme and fractions obtained during the purification. Some interesting conclusions can be derived from these results (Table 2-6). After anion exchange chromatography, the major β-keto ester reductase activity present afforded the 3*S* enantiomer and the *anti* diastereomer, in line with the notion that α acetoxy ketone reductase was the major reductase activity. However, further purification (gel filtration) revealed that a second enzyme with opposite stereoselectivity was also present at this stage, although it was later removed by size-exclusion chromatography. From previous reports, it was known that a D-enzyme co-eluted with α-acetoxy ketone reductase after anion exchange chromatography.[107] The elution profile from the gel filtration column indicated that this D-enzyme has a larger molecular weight than α acetoxy ketone reductase. This may indicate that fatty acid synthetase was present in enzyme fraction. In addition, the results obtained with α substituted β keto esters indicated that L-enzymes yielding both the *syn* and the *anti*

Stereoselectivity and Substrate Specificity of the Isolated Enzyme

The substrate specificity and the stereoselectivity of the isolated [redacted] reductase were assayed with several substrates (Table 2.6). The enzyme was [redacted] rednce acetoacetol to 1 acetoxy 2-propanol suggesting that the enzyme was indeed [redacted] acetoxy ketone reductase. Only one enzyme from baker's yeast capable of reducing this substrate has been reported.[110] This group also showed that the same enzyme [redacted] possessed the capability of reducing β keto esters to yield the 3S enantiomer and the [redacted] diastereomer.[109,110] The results presented here strongly argue that the isolated enzyme is the same as that isolated by Nakamura.

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diastereomers were present in the fraction obtained after anion exchange chr[illegible]

The formation of the *anti* diastereomer cannot be due to α acetoxy ketone re[illegible]e since the two peaks of activity eluting from the gel filtration column were clearly separated. The second peak eluting from the gel filtration column showed the activity assigned to α acetoxy ketone reductase. It resulted in formation of the *3S* enantiomer from reduction of ethyl acetoacetate and only the *anti* diastereomer when ethyl α allyl aceto[illegible]e is used as substrate. However the results obtained with ethyl α-propargyl acetoacetate indicate that an L enzyme forming the *syn* diastereomer is still co-eluting with the α acetoxy ketone reductase. These results underscore the complexity of this system and the difficulties inherent in assigning catalytic activities to specific enzymes.

As a summary after the gel filtration step the presence of at least three [illegible] reductases was revealed. In the first peak a D-enzyme and an L-enzyme yielding the *anti* diastereomer were present. The low de associated with the reduction of α substituted β keto esters suggested the presence of third reductase yielding the *syn* diastereomer. This enzyme activity was also present in the second peak co-eluting with α-acetoxy ketone reductase from which it was separated after the affinity columns. The presence of this third reductase eluting along both peaks of activity may explain the differences in de associated with the different substrates a fact that could not be explained by the presence of only two reductases. The results associated with the reduction of the β lactam derivative further confirmed the notion that several enzymes were still present in both fractions obtained from the gel filtration chromatography.

The final purification step yielded an enzyme fraction with the activity attributed to α-acetoxy ketone reductase; however the results obtained with ethyl α-propargyl

acetoacetate indicated that [illegible] eliminating activity [illegible] fraction. As stated earlier this fraction also reduced acetoacetol to 1,3-butanediol further confirming the assignment of this enzyme.

Table 2-6 Substrate specificity and stereoselectivity of the isolated reductase and different fractions along the purification protocol

Substrate	Purification Step			
	DEAE (anion exchange)	Gel filtration (peak 1)	Gel filtration (peak 2)	Matrex Red (300 fold purified)
	>98 % ee (*S*)	26.5 % ee (*S*)	>98 % ee (*S*)	>98 % ee (*S*)
	>98 % de[1] (*anti*)	25 % de[1] (*anti*)	>98 % de (*anti*)	>98 % de (*anti*)
	not determined	41 % de (*anti*)	14 % de (*anti*)	98 % de (*anti*)
	3*R*4*R* – 26 % 3*S*4*S* – 0 % 3*S*4*R* – 18 % 3*R*4*S* – 56 %	3*R*4*R* – 0 % 3*S*4*S* – 14 % 3*S*4*R* – 80 % 3*R*4*S* – 6 %	3*R*4*R* – 41 % 3*S*4*S* – 0 % 3*S*4*R* – 11 % 3*R*4*S* – 48 %	No conversion[2]
	not determined	not determined	not determined	1-acetoxy-2-propanol

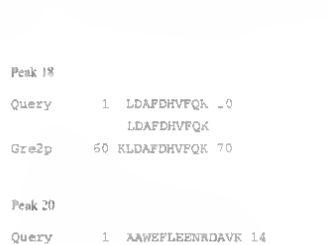
[1] The enantioselectivity for reduction of α substituted β-keto esters was always >98 % ee

[2] Complete conversion was not achieved

Sequencing of the α Acetoxy Ketone Reductase and Identification of the Gene Encoding this Activity

The protein preparation from the Matrex Red A column showed a major protein band whose presence correlated with the reductase activity. Fractions 35 and 36 containing the greatest amount of enzyme activity were pooled together, concentrated and used for sequencing. The presence of two minor contaminants required further purification of the major band by gel electrophoresis. The band of interest was then blotted into a PVDF membrane and sent to the University of Florida Protein Core for N-terminal sequencing. The sequencing results indicated that the protein was N terminally blocked since no sequence was being detected and the amino acid analysis indicated that the amount of protein was sufficient to provide sequencing data. Based on these results another sample was prepared and submitted to the Protein Core for internal sequencing. A sample containing 400 ng of protein was purified in a SDS polyacrylamide gel from which the major band was cut out of the gel. The sample was treated with a Lys-C protease and the resulting peptides were separated by HPLC. Three fragments were sequenced and the results obtained were compared with the *Saccharomyces cerevisiae* genome database. In all three cases the Gre2p protein presented almost complete identity with the sequencing results (Figure 2-7). In addition, each region of homology followed a lysine residue which was in agreement with the hydrolysis protocol employed. From these results the α-acetoxy ketone reductase was identified as the Gre2p encoded by the nuclear gene *GRE2*. A BLAST search indicated strong similarity between Gre2p and plant dihydroflavonol

and cinnamoyl CoA reductases, both members of the short-chain alcohol dehydrogenase superfamily.



1 Residues marked X could not be identified due to instrument malfunction.

Figure 2-7 Comparison of the sequencing data obtained for α acetoxy ketone reductase and enzymes in the *S. cerevisiae* genome database.

Conclusions

The α acetoxy ketone reductase activity has been previously assigned to the hypothetical protein encoded by the YJR105w ORF. The experimental results presented here demonstrated conclusively that this assignment was incorrect. The publication and

sequencing of the α acetoxy ketone reductase protein indicated that this enzyme was encoded by the *GRE2* gene. The Gre2p was N-terminally modified which may have led to the previous error the sequence obtained was likely due to some contamination in the in the same sample.

The correct identification of the gene encoding for the α acetoxy ketone reductase activity was essential for the progress of the overall project since it provided the last piece of information needed to construct the modified yeast strains. As presented earlier the genes encoding fatty acid synthetase (*FAS1 FAS2*) and aldo keto reductase (*FRI*) have been identified. The characterization of *GRE2* allowed the construction of single double the over-expression systems and knock out of each of these reductases and the evaluation of their performance on whole cell mediated reductions of β-keto esters.

CHAPTER 3
CONSTRUCTION OF THE FIRST GENERATION OF ENGINEERED YEAST STRAINS AND THE ASSOCIATED IMPROVEMENTS IN ENANTIO- AND DIASTEREOSELECTIVITY

The identification of the genes encoding fatty acid synthetase, aldo keto reductase and oxoacetoxy ketone reductase allowed the design of a first generation of yeast strains with altered stereospecificities. Two basic genetic tools were used to manipulate the enzyme expression levels in yeast and thus the relative catalyst's concentration: gene over-expression and gene knockout (Figure 3-1).

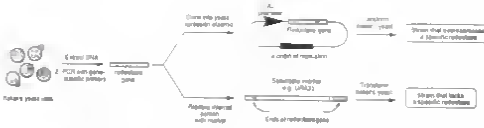


Figure 3-1 General strategy for the genetic design of the first generation of engineered yeast strains with altered stereospecificity

Strains over-expressing each of these reductases were constructed and analyzed for changes in the enantio- and diastereoselectivities of β-keto ester reductions. The over expression of a particular enzyme changed the relative catalyst concentrations inside the cell, which would be expected to affect the stereoselectivity of the designed strain. In a parallel approach, mutant strains lacking each one of these reductases were also obtained

The absence of the competing enzyme in the new strain should also result in altered stereoselectivity

The results obtained from this first generation of engineered strains provided good improvements in the stereoselectivities of baker's yeast reductions however incomplete stereoselectivity was still a common outcome of these biotransformations The results obtained with the mutant strains uncovered the presence of more reductases in baker's yeast that participate in the reductions of β keto esters A comparative analysis of the results provided a clearer picture of the role of each reductase in whole cell-mediated reduction of β keto esters by baker's yeast and suggested methods to further improve the stereoselectivity

Over Expression of Fatty Acid Synthetase, Aldo Keto Reductase and α Acetoxy Ketone Reductase and Associated Effects on Whole Cell-Mediated Reduction of β Keto Esters

The development of strains with increased expression for each of these reductases was achieved by transformation of the parental strains with vectors designed to over express each enzyme For fatty acid synthetase and aldo keto reductase, the systems were based on *S. cerevisiae* expression vector pYES2,[138] while the expression system for α acetoxy ketone reductase was based on pAAH5 [128] Two *S. cerevisiae* strains InVSc1 and 15C were initially used although most of the later work focussed on 15C Strains carrying the over-expression plasmids were obtained by transformation of these parental strains by the high efficiency lithium acetate protocol [147]

Choice of Expression Vectors for the Over Expression of Yeast Reductases

Different types of yeast expression systems have been devised by several groups and provide the starting point for regulated protein expression in yeast [139,14] These

different systems vary in terms of stability level of protein expression and regulation of the expression. Three different types of vectors present very different copy numbers which affects both the stability and the level of expressed protein. Higher copy number is associated with larger protein production but also lower stability. Thus the best level of protein expression results from a compromise between these two factors. The most stable way to maintain introduced genes is by integration of the entire plasmid into the chromosome by homologous recombination. The gene is then present in only one copy which results in very low level of expression but provides a very stable system [14]. The ARS-CEN vectors also present great stability due to the presence of a centromere locus. These vectors are maintained at a copy number of 1-2 per cell, resulting in low to mid level of protein production, but they present the advantage of very high plasmid retention (97 % after 20-30 generations in non selective media) [39]. The 2μm vector generally provides the highest level of protein production since it is present in an average number of 10-40 per cell. However, since high expression of a protein can be deleterious for the cell, these plasmids may suffer from low stability and the strain may need to be grown in a selective media to maintain the plasmid [39]. The vectors selected for this project were 2μm vectors since a high level of expression of the desired enzyme would more likely overcome the competition by other reductases present in yeast. ARS-CEN systems were used as an alternative when stability problems arose, as was the case with over expression of fatty acid synthetase.

The type of promoter present on the system also regulates the level of expression and vectors with both constitutive and inducible promoters are available [11,13,14]. An inducible promoter offers the advantage that expression of the enzyme can be controlled

by changes in growth conditions thus the enzyme can be produced only when needed for the biotransformation Among inducible promoters *GAL*, *PGK* and *ADH1* respond to the carbon source, while the *PHO5* promoter responds to the inorganic phosphate concentration [38 142] The *GAL* promoter is activated in the presence of galactose while the *PGK* and *ADH1* respond to the presence of glucose The vectors used along this work contained either the *GAL* promoter or the *ADH1* promoter the selection of different promoters for different genes was based on the finding that the choice of carbon source affected the endogenous level of competing yeast reductases

Effect of different carbon sources on whole cell mediated reductions of β-keto esters

Control experiments performed with unmodified yeast cells in media containing either glucose or galactose demonstrated that the change in carbon source had a strong effect on the outcome of the biotransformation This effect was first observed with test substrates and an extended analysis showed that it was also observed in all the other substrates used in this study The outcomes of these experiments dictated the choice of vector to be used for the over-expression of the different enzymes so that the natural effect of the carbon source would add to the desired stereoselectivity of the targeted protein. Whether the carbon sources induced production of endogenous yeast enzymes with similar stereoselectivities or repressed production of unwanted competitors was not explored in detail

The outcomes of biotransformations with the yeast strain 15C in the presence of the two carbon sources is summarized in Table 3 1 The presence of galactose in the media resulted in larger production of the *R*-enantiomer which provided a good framework for the over-expression of the D-enzyme fatty acid synthetase The larger

effect of the change in carbon source was observed in the reduction of ethyl 2-ethyl acetoacetate. In this case, the diastereoselectivity was shifted from 59 % de *anti* to 71 % de *syn* when glucose was replaced by galactose (entry 8). A similar effect was observed with ethyl 2-ethyl acetoacetate (entry 7). These results indicated that galactose grown cells provided the best setting for the over-expression of aldo keto reductase since this enzyme also afforded the *syn* diastereomer from these substrates. Similar considerations resulted in the choice of glucose as the carbon source for over-expression of the enzyme producing the *anti* diastereomer, α-acetoxy ketone reductase. Based on these results, vectors containing the GAL promoter were used for the over-expression of aldo-keto reductase and fatty acid synthetase while the expression system for α-acetoxy ketone reductase was based on pAAH5, which contains the ADH1 promoter.

33

Figure 3-2. Substrates used for testing the effect of the carbon source on the whole cell yeast-mediated β-keto ester reductions

Table 3-1 Effect of the carbon source on the stereoselectivity of whole cell-mediated yeast reduction of β-keto esters

Entry	Substrate (33)			Carbon source	
	R_1	R_2	R_3	Glucose	Galactose
1	Me	H	Me	90 % ee (*S*)	84 % ee (*S*)
2	Et	H	Me	70 % ee (*R*)	86 % ee (*R*)
3	Me	H	Et	95 % ee (*S*)	84 % ee (*S*)
4	Et	H	Et	8 % ee (*R*)	70 % ee (*R*)
5	Propyl	H	Et	>98 % ee (*R*)	>98 % ee (*R*)
6	Me	Me	Et	>98 % de (*syn*)	83 % de (*syn*)
7	Me	Et	Et	10 % de (*syn*)	59 % de (*syn*)
8	Me	Allyl	Et	59 % de (*anti*)	17% de (*syn*)
9	Me	Propargyl	Et	30 % de, (*syn*)	28 % de (*syn*)

Vectors used as the base of the over-expression systems

Based on all the previous considerations, the 2μm vectors pYES2 pJS351 and pAAH5 (Figure 3-3) were used as the frameworks for constructing the required expression systems. The Invitrogen plasmid pYES2 carries the GAL promoter, the 2μm origin of replication, and the *URA3* cassette for selection in *S. cerevisiae* strains auxotrophic for uracil. Plasmid pJS351 was derived from pYES2 by replacing the *URA3* marker with *HIS3* and this construct was prepared by Dr. J. Stewart. Plasmid pAAH5 is also a high copy number plasmid that carries the *LEU2* cassette for selection in yeast leucine auxotrophic strains and gene expression is controlled by the ADH1 promoter which is induced by the presence of glucose instead of galactose as in the previous ones. This plasmid was generously provided by Dr. G. Ammerer [99]. All these vectors contain the ampicillin resistance gene (*bla*) and the *colE1* origin of replication for maintenance and cloning in *E. coli*.

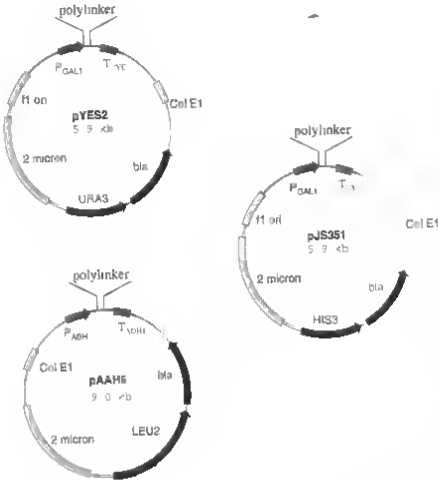


Figure 3 3 Vectors pYES2, pJS351 and pAAH5 used as frameworks for the construction of the yeast reductases expression systems

Construction of the Over Expression Systems for Fatty Acid Synthetase

The over-expression of fatty acid synthetase required the construction of two expression plasmids one for each subunit (α and β) The genes encoding both subunits (*FAS2* and *FAS1* respectively) were available in plasmids pJS222 and pJS229 generously provided by Dr E Schweizer Construction of several intermediate plasmids was

necessary to allow the final subcloning of the genes into pYES2 and pJS351 to furnish the final expression constructs pSRG13 and pSRG15. The low stability associated with these constructs prompted the subsequent design of ARS CEN expression vectors. To construct these the cassettes for *FAS1* and *FAS2* were subcloned into YCplac vectors (Figure 3-4) providing expression plasmids pSRG24 and pSRG22.

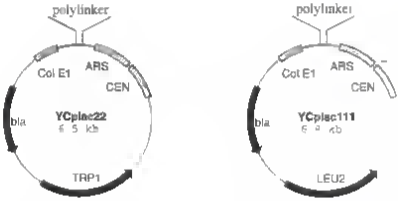


Figure 3-4. Vectors YCplac22 and YCplac111 used in the construction of the fatty acid synthetase ARS CEN expression plasmids.

Expression system based on the 2μm vector

The construction of the first set of expression plasmids for fatty acid synthetase is summarized in Figures 3-5 and 3-6. Plasmids pJS229 and pJS222 were used as templates for PCR amplifications of fragments corresponding to the beginning and end of each gene. This strategy avoided the amplification of large fragments with the increased likelihood of introducing mutations and it allowed the incorporation of flanking *Xho*I and

*Sac*I sites required for the final subcloning. Then the genes were reconstructed in pBC SK(+) by subcloning each of the PCR products and incorporating the non amplified region of the gene. Finally *FAS1* and *FAS2* were subcloned into pYES2 and pJS351 resulting in pSRG13 and pSRG15 respectively.

This strategy required two PCR amplifications and the construction of three intermediate plasmids for obtaining each of the final expression plasmids. The beginning of the *FAS1* gene was PCR amplified and incorporated as a *Sac*I, *Pst*I fragment in pBC SK(+) resulting in pSRG6. The presence of the *Xho*I site downstream from the *Pst*I site provided the framework for linking this piece to the other two fragments of the gene. The 3' end of the *FAS1* gene was PCR amplified and subcloned as a *Hpa*I, *Xho*I fragment into pBC SK(+), furnishing pSRG4. The presence of the *Bgl*I site in the cloned fragment was used for subcloning it, along with the *Bgl*I, *Pst*I internal fragment of *FAS1* into pSRG6 resulting in pSRG10. This final plasmid contained the complete *FAS1* gene with appropriate flanking restriction sites for subcloning the *FAS1* gene into pYES2 to afford pSRG13 (Figure 3-5).

A similar strategy was used for cloning *FAS2* into pJS351 (Figure 3-6). In this case, cloning of the 3' portion of the gene between the *Pst*I and *Xho*I sites of pBC SK(+) provided the needed framework for subcloning the complete gene. The presence of the *Sac*I site upstream of the *Pst*I site in pSRG9 allowed incorporating the two other fragments of the gene. The 5' region of the gene was PCR amplified and cloned into pBC SK(+), furnishing pSRG8. It was then released from pSRG8 as a *Sac*I, *Acc*I fragment and subcloned, along with the internal *Acc*I, *Pst*I segment from pJS222, into pSRG9 to

furnish pSRG12. The presence of the *Sac*I and *Xho*I sites flanking the gene in this final plasmid allowed for subcloning *FAS2* into pJS351 to obtain pSRG15 (Figure 3.6).

The presence of different selectable markers in pSRG13 and pSRG15 allowed introducing both plasmids into the same strain. Thus transformation of InvSc1 resulted in the final yeast strain InvSc1 (pSRG13, pSRG15). However, the presence of *HIS3* as the auxotrophic marker in pSRG15 limited its use to InvSc1 since 15C does not carry *his3* mutation. In order to over-express fatty acid synthetase in this strain, a new expression vector carrying the TRP1 auxotrophic marker was constructed. The cassette for expression of the *FAS2* gene under control of the GAL promoter was extracted from pSRG22 with *Sal*I and *Bam*HI, then subcloned between the same restriction sites of YEplac112 resulting in pSRG29 (Figure 3.7). This plasmid along with pSRG13 was used to transform 15C furnishing the strain 15C(pSRG13, pSRG29) for the over expression of fatty acid synthetase.

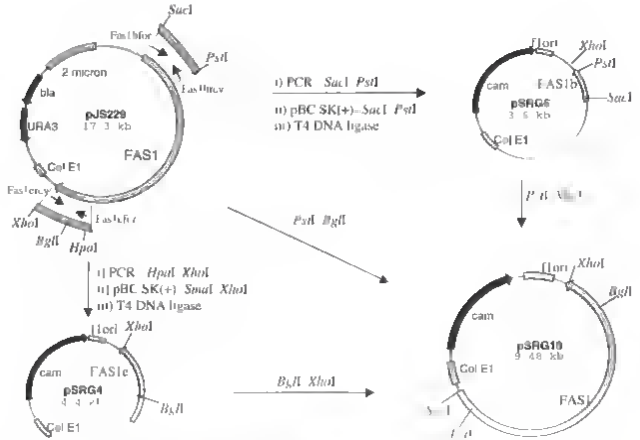


Figure 3.5 Construction of the over expression system for FAS1 pSRG13

Figure 3.5 continued

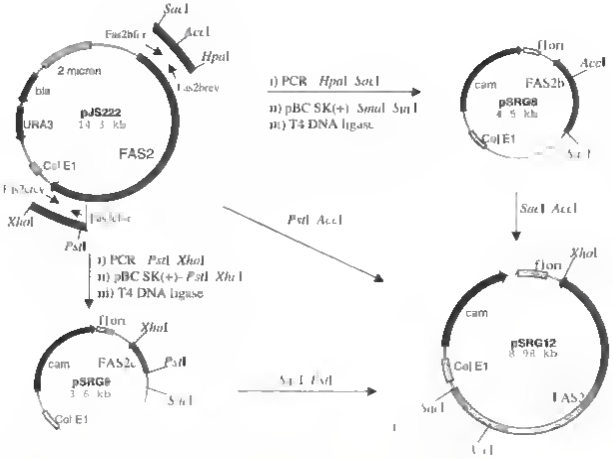


Figure 3 6 Construction of the over expression system for *FAS2* pSRG15

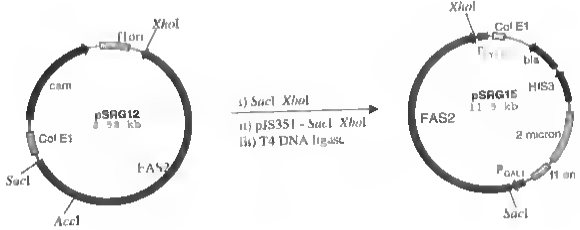


Figure 3.6 continued

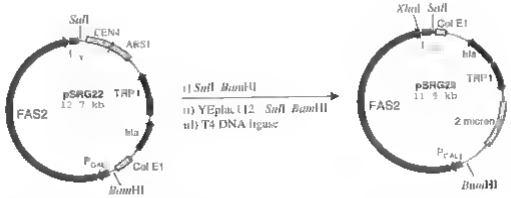


Figure 3.7 Construction of the over expression system for *FAS2* with a *TRP1* selectable marker pSRG29

Expression system based on ARS-CEN vectors

The construction of the ARS CEN based expression plasmids for *FAS1* and *FAS2* is summarized in Figures 3 8 and 3 9 The absence of any promoter and terminator sequences in the YCplac vectors series required subcloning the whole cassette including the GAL promoter the gene (*FAS1* or *FAS2*) and the CYC1 terminator

The subcloning strategy for the *FAS1* cassette involved the construction of two intermediate plasmids before the final expression vector pSRG24 could be prepared The major difficulty associated with this operations was the need for compatible restriction sites flanking the entire expression cassette A pBC SK(+) derivative containing the CYC1 terminator (pJS346) had been previously prepared by Dr J Stewart and was used as the base for creating pSRG21 (Figure 3 8) The GAL promoter and the *FAS1* gene were isolated from pSRG13 as *Spe*I *Pst*I and *Pst*I *Sph*I fragments respectively Ligation of these two fragments into the *Spe*I *Sph*I sites of pJS346 resulted in pSRG21 Note that the presence of an internal *Spe*I site within *FAS1* required this complex method In this vector the *FAS1* cassette containing both promoter and terminator was flanked by *Bss*HII sites A YCplac111 derivative was therefore created with a *Bss*HII site within the polylinker by introducing an oligonucleotide with the appropriate sequence between the *Kpn*I and *Eco*RI sites of the YCplac111 polylinker (Figure 3 8 cont) The resulting plasmid pSRG23 was used for subcloning the *FAS1* cassette extracted from pSRG21 as a *Bss*HII fragment providing the final expression vector pSRG24

The construction of pSRG22, an ARS CEN based expression system for *FAS2* was much simpler (Figure 3-9). The GAL promoter along with the gene was released from pSRG15 as a *Spe*I *Xho*I cassette. The CYC1 terminator was isolated as a *Xho*I, *Spe*I fragment from pJS347 (a vector containing the GAL promoter and CYC terminator that was prepared by Dr. J. Stewart). Both fragments were subcloned into the *Xho*I site of YCplac22 (since *Xho*I and *Spe*I generate compatible cohesive ends).

The generated ARS-CEN expression systems, pSRG22 and pSRG24, carried different selectable markers what allowed introducing both plasmids into the same strain. Transformation of 15C and InvSc1 resulted in two new strains for the expression of fatty acid synthetase: 15C (pSRG22, pSRG24) and InvSc1 (pSRG22, pSRG24). The new recombinant strains were very stable, and 87% of the cells carried both plasmids after 24 h of growth on non-selective media.

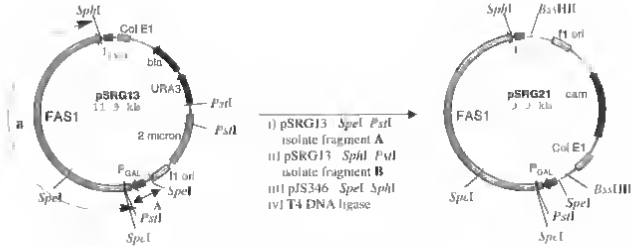


Figure 3.8 Construction of the ARS CEN vector for *FAS1* pSRG24 (continued)

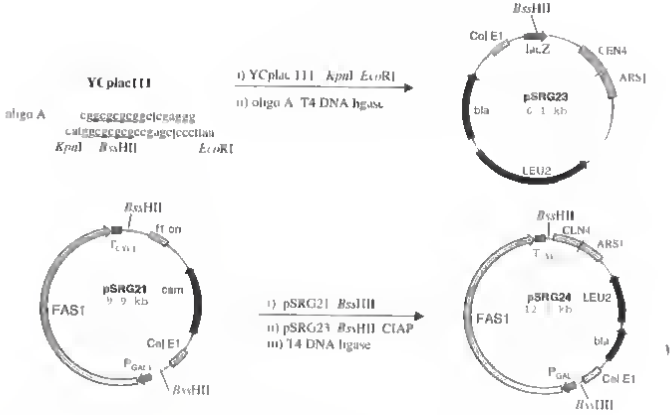


Figure 3-8 Construction of the ARS-CLN vector for *FAS1* pSRG24 (2nd part)

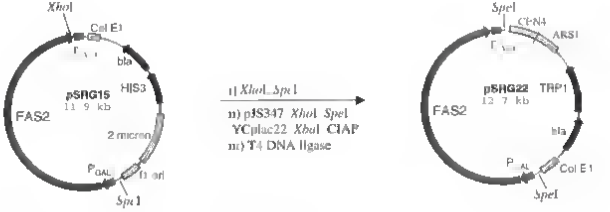


Figure 3) Construction of the ARS CEN vector for *FAS2* pSRG22

Construction of an Over Expression System for Aldo Keto Reductase

The expression plasmid for this enzyme (pSRG14) was derived from the yeast expression vector pYES2, and its construction is summarized in Figure 3-10. The *YPR1* gene was PCR amplified from *S. cerevisiae* genomic DNA with primers that incorporated suitable restriction sites for the cloning strategy. After amplification and digestion with *Sac*I and *Xba*I, the gene was cloned into these sites in pUC18, providing pSRG11. The expression plasmid, pSRG14, was created by subcloning the *YPR1* gene as a *Sac*I-*Sph*I cassette between the same sites in pYES2. This vector was used for transformation of 15C and InvSc1 resulting in engineered strains 15C(pSRG14) and InvSc1(pSRG14). Both strains exhibited good stability. In the case of 15C (pSRG14), 83 % of the cells retained the plasmid after 24 h of incubation under non selective conditions. This strain was chosen to probe the effect of aldo keto reductase over-expression on whole cell mediated reductions of β keto esters.

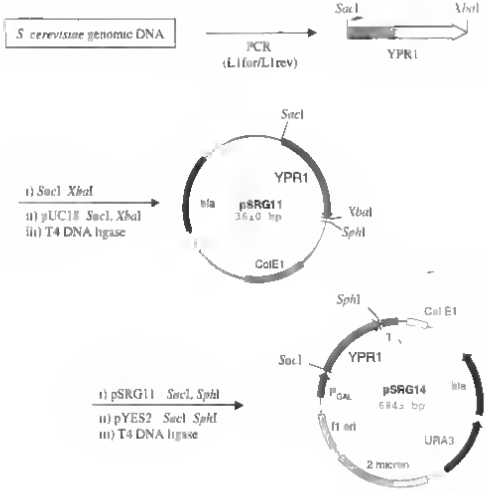


Figure 3-10 Construction of an expression plasmid for aldo keto reductase pSRG14

Construction of an Over Expression System for α Acetoxy Ketone Reductase

The expression plasmid for α-acetoxy ketone reductase (pSRG41) was derived from the yeast expression vector pAAH5 and its construction is summarized in Figure 3 11 The *GRE2* gene was PCR amplified from *S cerevisiae* genomic DNA with primers

that incorporated suitable restriction sites flanking the gene The originally-designed primers failed to amplify the *GRE2* gene so the reverse primer was then modified to anneal 70 bp after the end of the gene to avoid the possibility of hairpin loop formation present in the original primer After amplification and restriction enzyme digestion the gene was cloned as a *Hin*DIII cassette into the corresponding site of pAAH5 providing the expression vector pSRG41 This vector was used for transformation of 15C resulting in the engineered yeast strain 15C(pSRG41)

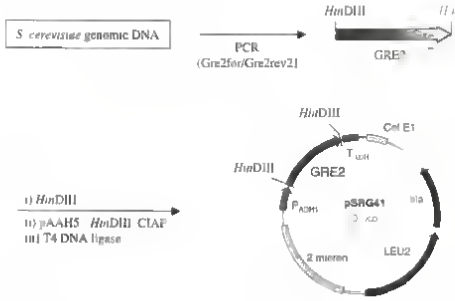


Figure 3-11 Construction of an expression plasmid for α-acetoxy ketone reductase pSRG41

Analysis of Whole Cell-Mediated β-Keto Esters Reductions by the Engineered Yeast Strains Over Expressing Each Reductase

The strains constructed for over-expression of fatty acid synthetase aldo keto reductase and α-acetoxy ketone reductase were tested for stereoselectivities in whole cell

mediated reduction of β-keto esters and the results were compared with those obtained with the unmodified strains. Two test substrates, ethyl 3-oxo-pentanoate (**33d**) and ethyl 2-allyl acetoacetate (**33h**), were used to test the improvements in enantio- and diastereoselectivities associated with the engineered strains since these substrates are reduced with poor selectivity by unmodified yeast cells. Strains over-expressing fatty acid synthetase were tested only for improvements in enantioselectivity of model substrate **33d** since α-substituted β-keto esters are not reduced by this enzyme.[137] The studies were then extended to a total of nine β-keto esters (Figure 3-12). Substrates **33a-g** were commercially available, while **33h** and **i** were synthesized by alkylation of ethyl acetoacetate with the corresponding alkyl bromide.

33

a R_1 = Me, R_2 = H, R_3 = Me
b R_1 = Et, R_2 = H, R_3 = Me
c R_1 = Me, R_2 = H, R_3 = Et
d R_1 = Et, R_2 = H, R_3 = Et
e R_1 = *n*-Pr, R_2 = H, R_3 = Et
f R_1 = Me, R_2 = Me, R_3 = Et
g R_1 = Me, R_2 = Et, R_3 = Et
h R_1 = Me, R_2 = allyl, R_3 = Et
i R_1 = Me, R_2 = propargyl, R_3 = E

Figure 3-12. Substrates used for testing the effect of the engineered strains on whole cell yeast mediated β-keto esters reductions.

Biotransformations were performed according to the conditions established earlier by our group.[143] The cells were prepared by growing them in YPD until the culture reached an OD_{600} between 4 and 6; under these conditions a high proportion of the population retained the plasmid. In cases where plasmid retention was a problem, the

cells were grown in minimum media with the appropriate supplements and 2% glucose The cells were then collected by centrifugation washed with buffer resuspended at a concentration of 0 1 g/mL in buffer with 15% glycerol and stored at –80 °C Biotransformations were performed in YP containing 2% of the desired carbon source that induced gene expression and 10 mM substrate. Freshly prepared or frozen cells were added to a final concentration of 2 mg/mL at the start of the reaction and the progress was monitored by GC Unless otherwise stated, the reactions were run to completion and the final enantiomeric and diastereomeric excess values were determined by chiral GC

Effect of Fatty Acid Synthetase Over Expression on the Enantioselectivities of Whole Cell-Mediated β-Keto Ester Reductions

The strains over-expressing fatty acid synthetase were tested for reduction of ethyl 3-oxo pentanoate and the results are presented in Table 3 2 As expected over-expression of fatty acid synthetase increased the amount of *R* enantiomer formed, which was the major isomer in all cases The associated improvement of 10 – 15 % in the enantiomeric excess although good, was lower than expected Despite the high stability associated with the ARS-CEN plasmids the strains carrying these plasmids gave lower improvements in enantioselectivity (entries 2 and 6) perhaps because of low levels of enzyme production The remaining strains carried high copy number plasmids which are usually associated with very effective enzyme expression Unfortunately this advantage was undermined by the low stability of these systems and only 12 % of the cells retained both plasmids after 24 h (the time required for the biotransformation entries 2 and 7)

A surprising result was obtained from InvSc1 (pSRG15) Despite the fact that this strain over-expressed only the fatty acid synthetase α subunit it gave a significant

improvement in enantioselectivity (entry 4). Considering the differences in plasmid retention, the improvement is comparable to that observed for InvSc1 (pSRG13, pSRG15). This result suggests that the α subunit is active on its own or that its over-expression induced the production of the β subunit. Biosynthetic control on the production of the fatty acid subunits has been suggested earlier [144].

Table 3-2. Effect of the over-expression of fatty acid synthetase on the enantioselectivity of ethyl 3-oxo pentanoate whole cell-mediated reduction.

Entry	Yeast strain	Plasmid retention	% e.e.
1	InvScI	---[1]	46
2	InvScI (pSRG22, pSRG24)	87 %	50
3	InvScI (pSRG13, pSRG15)	12 %	60
4	InvScI (pSRG15)	16 %	67
5	15C	---[1]	70
6	15C (pSRG22, pSRG24)	87 %	77
7	15C (pSRG13, pSRG29)	12 %	80

[1] Not applicable

The preliminary results indicated that the 2μm based plasmids provided the best over-expression systems. To overcome the problem of low plasmid retention observed during the biotransformations, the experiments were repeated in selective media. The cells were pre-grown in selective media to guarantee that most of the cells would contain the plasmids at the beginning of the biotransformation. Biotransformations were tried in both YP-galactose (non-selective media) and SD-galactose (selective media) and the results are presented in Table 3-3. The results from the previous experiments are also presented there for comparison (entries 2 and 5).

Growing the preculture in selective media had a profound effect on the outcome of the biotransformations clearly indicating that low plasmid retention during the preparation of the cells was a major problem. Interestingly the use of selective media during the biotransformation did not provide further improvements which seemed to have a limit of 88 % ee (entries 3 4 and 7 8) This could be caused either by low intrinsic enantioselectivity of fatty acid synthetase towards this substrate or by very tight control of FAS expression in yeast, that limited its total production

Table 3 3 Effect of the over-expressing fatty acid synthetase on the enantioselectivity of ethyl 3-oxo pentanoate whole cell-mediated reductions Influence of different growth media.

Entry	Yeast strain	Preculture media	Biotransformation media	% ee
1	InvScI	YPD	YP-Gal	46 %
2	InvScI (pSRG13 pSRG15)	YPD	YP Gal	60 %
3	InvScI (pSRG13 pSRG15)	SD	YP Gal	88 %
4	InvScI (pSRG13 pSRG15)	SD	SD-Gal	88 %
5	15C	YPD	YP-Gal	70 %
6	15C (pSRG13 pSRG29)	YPD	YP-Gal	80 %
7	15C (pSRG13 pSRG29)	SD	YP-Gal	88 %
8	15C (pSRG13 pSRG29)	SD	SD-Gal	88 %

Despite the stability problems associated with systems over-expressing fatty acid synthetase some improvements in the enantioselectivity were achieved. Moreover the results obtained by over-producing only the α subunit suggested a simpler strategy that could still give useful improvements in stereoselectivity However since many problems were associated with the over-expression of fatty acid synthetase it was considered that better results might be possible by knocking out competing enzymes

Effect of Aldo Keto Reductase Over Expression on the Stereoselectivities of Whole Cell Mediated β Keto Ester Reductions

Strain 15C (pSRG14) over expressing aldo keto reductase was used for the reductions of several β-keto esters and the results are presented in Table 3-4 As expected, this strain produced significantly more (*S*)-alcohol than the unmodified strain It also provided very high enantioselectivities in the reductions of **33a** and **33c** (entries 1 and 3) Reduction of the other non substituted β-keto esters with this strain still yielded the (*R*) alcohols as the major product (entries 2, 4 and 5) although the over expression of aldo keto reductase at least doubled the production of the (*S*)-alcohol for substrates **33b** and **d**, which constitutes a significant change The effects of altering the level of aldo-keto reductase were demonstrated more clearly when α-substituted-β keto esters were used as substrates, since this enzyme produces the *syn* diastereomers whereas others (primarily α-acetoxy ketone reductase) afford the *anti* alcohols As expected the aldo-keto reductase over-expression strain resulted in a high production of the *syn* diastereomer from substrates **33f-i** (entries 6 and 9) The increase in diastereomeric excess compared to the control was higher for larger α substituents and reduction of **33i** with this strain yielded only one of the four possible diastereomers nicely illustrating the power of the engineered yeast strategy

Effect of α Acetoxy Ketone Reductase Over Expression in the Stereoselectivity of Whole Cell Mediated β Keto Ester Reduction

The strain over-expressing α acetoxy ketone reductase 15C (pSRG41) was tested for the reductions of several β keto esters and the results are presented in Table 3 5 The

over-expression of this enzyme had a large impact on the outcomes of whole cell mediated reductions, favoring the production of (*S*)-alcohol and the *anti* diastereomer

Table 3-4 Effect of aldo keto reductase over-expression on whole cell-mediated reduction of β-keto esters

Entry	Substrate (33)				Strains	
	33	R_1	R_2	R_3	15C	15C (pSRG14)
1	**a**	Me	H	Me	84 % ee (*S*)	93 % ee (*S*)
2	**b**	Et	H	Me	86 % ee (*R*)	36 % ee (*R*)
3	**c**	Me	H	Et	84 % ee (*S*)	>98 % ee (*S*)
4	**d**	Et	H	Et	70 % ee (*R*)	36 % ee (*R*)
5	**e**	Propyl	H	Et	>98 % ee (*R*)	97 % ee (*R*)
6	**f**	Me	Me	Et	83 % de (*syn*)	96 % de (*syn*)
7	**g**	Me	Et	Et	59 % de (*syn*)	81 % de (*syn*)
8	**h**	Me	Allyl	Et	17% de (*syn*)	65 % de (*syn*)
9	**i**	Me	Propargyl	Et	28 % de, (*syn*)	>98 % de (*syn*)

Only one enantiomer was obtained from the reduction of **33a** and **c** providing a very useful biocatalyst for reductions of these two substrates An even more impressive improvement was obtained with substrates **33b** and **d**, where the stereoselectivities of the reactions were shifted to yield the (*S*)-alcohol as the major products with enantiomeric excess values that showed that five times more (*S*)-enantiomer was produced In the case of ketone **33e** the (*R*)-alcohol was the major product upon reduction with the engineered strain, however the production of 32 % (*S*)-enantiomer was achieved from a substrate that was only reduced to the (*R*) alcohol by unmodified baker s yeast

The effects of increasing the level of α-acetoxy ketone reductase in 15C (pSRG41) were also observed with α-substituted β-keto esters With the exception of substrate **33f** the *anti* diastereomer was the major product from all of these

biotransformations. The results with substrate **33f** were not surprising since previous reports indicated that purified α-acetoxy ketone reductase reduced this substrate to yield the *syn* diastereomer in 64-80 % de [60,108]. Similarly to the results obtained with aldo keto reductase, stronger effects on the diastereoselectivity were associated with larger α substituents. The reduction of **33b** and **i** with this strain resulted in formation of only the 2*S*, 3*S anti* diastereomer, introducing two chiral centers in a single reaction.

Table 3.5. Effect of α-acetoxy ketone reductase over-expression on whole cell mediated reductions of β-keto esters.

Entry	Substrate (33)				Strains	
	33	R_1	R_2	R_3	15C	15C (pSRG41)
1	**a**	Me	H	Me	90 % ee (*S*)	>98 % ee (*S*)
2	**b**	Et	H	Me	70 % ee (*R*)	33 % ee (*S*)
3	**c**	Me	H	Et	95 % ee (*S*)	>98 % ee (*S*)
4	**d**	Et	H	Et	8 % ee (*R*)	81 % ee (*S*)
5	**e**	Propyl	H	Et	>98 % ee (*R*)	34 % ee (*R*)
6	**f**	Me	Me	Et	>98 % de (*syn*)	71 % de (*syn*)
7	**g**	Me	Et	Et	10 % de (*syn*)	38 % de (*anti*)
8	**h**	Me	Allyl	Et	59 % de (*anti*)	>98 % de (*anti*)
9	**i**	Me	Propargyl	Et	30 % de (*syn*)	>98 % de (*anti*)

In addition to the important improvements in stereoselectivity associated with the over-expression of α-acetoxy ketone reductase, the rates of the biotransformations were also affected significantly. The examples presented in Figure 3.13 nicely illustrate how the over-expression of this enzyme resulted in an increased rate of reduction and the reactions were completed in less than half the time required for control strains. The presence of the ADH1 promoter in this system compared to the GAL promoter in the previous ones could explain this different rate of conversion. The over-expression from a

on with the ADH1 promoter starts earlier than that one with the GAL promoter since the latter presents a lag period of around six hours [39]

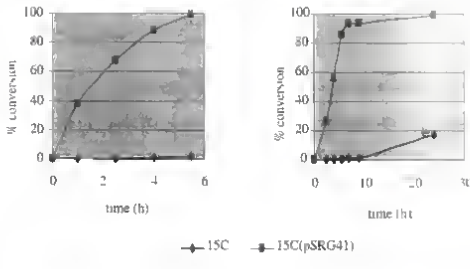


Figure 3.13 Comparative rate of β keto ester reduction by the designed yeast 15C (pSRG41) and the parental strain 15C

Strains Carrying Deletions of each Reductase and Analysis of the Associated Effects on Whole Cell-Mediated Reductions of β Keto Esters

A parallel approach to the creation of yeast strains with improved stereoselectivity was based on the use of mutants that lacked a specific reductase activity. The absence of one of the competing enzymes should affect the outcome of the reaction. The mutant strains were analyzed for changes in the enantio- and diastereoselectivities of β keto ester reductions with the same set of substrates used to test their over-expression. The results

obtained from these mutants also helped in understanding the role that each enzyme plays in whole cell mediated reductions of β keto esters

Obtaining Mutant Strains Defective in Fatty Acid Synthetase, Aldo Keto Reductase and α Acetoxy Ketone Reductase

A strain defective in fatty acid biosynthesis with a mutation previously shown to be associated with the reductase activity of fatty acid synthetase was obtained from the American Type Culture Collection (ATCC26403) [94] This mutation was not characterized further and the strain was used in the initial phases of these studies A complete *Δfas2* deletion was utilized after it became commercially available since these strains offer a genetically stable and well-defined background. This strain was obtained from Research Genetics as a diploid A haploid derivative (2B) was obtained by sporulation of the diploid A 15C mutant carrying a deletion of the *YPR1* gene was constructed by direct gene replacement and its construction is described below Finally the mutant strain carrying a deletion of the *GRE2* gene was purchased from the Euroscarf consortium.

Construction of 2B, a *Δfas2* Haploid Strain

This strain was obtained by sporulation of a heterozygous diploid strain followed by spore dissection and screening for colonies unable to grow in the absence of exogenous fatty acids The diploid strain 21061 carries a deletion of one of the *FAS2* alleles in which the gene was replaced by the cassette encoding geneticin resistance This strain was first plated in pre-sporulation media for 24 h then subcultured onto sporulation media. Spores developed after 15 18 days A very low efficiency of sporulation was detected and only 10 % of the cells formed spores The experiment was repeated three times with the same outcome Five ascospores were dissected through micromanipulation

and plated to YPD supplemented with fatty acids The master plate containing the spore progeny was replica plated onto two different selective media for screening The spores carrying the *fas2 kanMX4* gene replacement were able to grow in the presence of geneticin but were unable to grow in the absence of fatty acids Spore 2B exhibited the expected phenotype and was selected for studies on the effect of this mutation on β keto esters reductions and the results obtained in those studies (*vide infra*) further confirmed the Δ*fas2* phenotype

Construction of a Strain Defective in Aldo Keto Reductase

A 15C mutant strain carrying a deletion of the *YPR1* gene that encodes aldo keto reductase was created by the micro-homology PCR mediated gene disruption [131 145 146] Two PCR primers were designed in which the sequence of the first 35 bases was derived from the beginning or and of the *YPR1* gene while the remainder was designed to amplify a *URA3* cassette These primers were used for PCR amplification of the *URA3* cassette resulting in a linear DNA fragment containing the *URA3* cassette flanked by 35 bases of sequence homology to the ends of the *YPR1* gene Transformation of 15C with this PCR product was performed by the high efficiency lithium acetate protocol with selection for uracil prototrophy [147] The low frequency of the micro-homology recombination event required larger amounts of DNA than those normally used with this transformation technique. Transformation with 90 450 and 900 ng of DNA resulted in one seven and 18 transformants respectively

The new strains were first assayed for the ability to reduce ethyl α-allyl acetoacetate to detect those unable to produce aldo keto reductase Most transformants reduced this compound with a stereoselectivity similar to the parental strain (60 % de

ura3) presumably reflecting illegitimate recombination however 25 % of the screened colonies produced more *ura3* diastereomer (83 % de, *ura3*) suggesting the absence of aldo keto reductase

Mutant strain JS3 was characterized for presence of the desired knock out and the absence of the wild type allele by both PCR and Southern blot techniques Genomic DNA was isolated from JS3 for the characterization of the deletion and from 1SC the parental strain for the control experiments

PCR characterization of the JS3 mutant strain

The first set of primers (L1for-*YPR1*seq2f) was designed to amplify a segment of wild type *YPR1* the second set (L1for L1rev) was designed to indicate the replacement of the wild type allele by *ypr1*::*URA3* by restriction enzyme analysis of the PCR product (Figure 3-14)

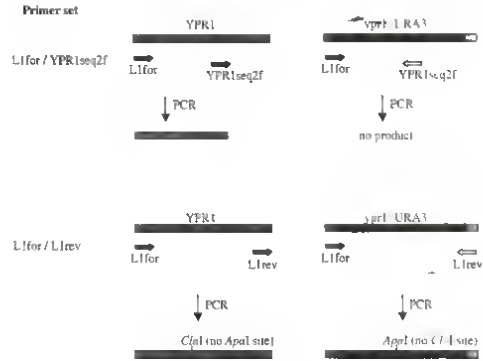


Figure 3-14 Characterization of JS3 (15C(*ypr1 URA3*)) based on PCR experiments

The results obtained from the PCR experiments with the two primer sets are summarized in Table 3-6. The first set of primers amplified a 700 bp fragment of the *YPR1* gene when genomic DNA prepared from the parental strain (15C) DNA was used as template; substitution of the PCR template by JS3 genomic DNA resulted in no product formation indicating that the *YPR1* wild type allele was not present in JS3. The gene replacement was confirmed by using the set of primers originally designed to PCR amplify the *YPR1* gene (L1for L1rev). These primers amplified both the wild type allele and the deleted version of the gene resulting in PCR products of similar sizes. The difference in restriction enzyme patterns was used to identify the nature of the PCR products. Digestion with *ClaI* produced two fragments of 430 and 540 bases from the

15C PCR product while it left the JS3 product uncut. The reverse result was observed when both fragments were incubated with *Apa*I thus enzyme cut the JS3 product but left the 15C product intact. All these results indicated that the *YPR1* gene deletion was successful in the JS3 mutant strain

Table 3-6 Characterization of JS3 (15C(ypr1 URA3)) based on PCR experiments

Primer set		Enzyme digestion	Template for the PCR experiment	
			15C	JS3
L1for	YPR1seq2f	None	700 bp	no product
L1for	L1rev	*Cla*I	400 + 560 bp	1050 bp
L1for	L1rev	*Apa*I	970 bp	450 + 600 bp

Southern blot characterization of the JS3 mutant strain

Two southern blot probes were employed to further verify the gene disruption The first probe targeted the internal fragment of *YPR1* that was replaced by *URA3* in JS3 this probe would indicate presence or absence of the wild type allele (Figure 3-13) Probe II was designed to bind the *URA3* gene that replaced the *YPR1* gene (Figure 3-15) the restriction enzyme pattern will indicate whether the *URA3* gene was present in the *YPR1* loci

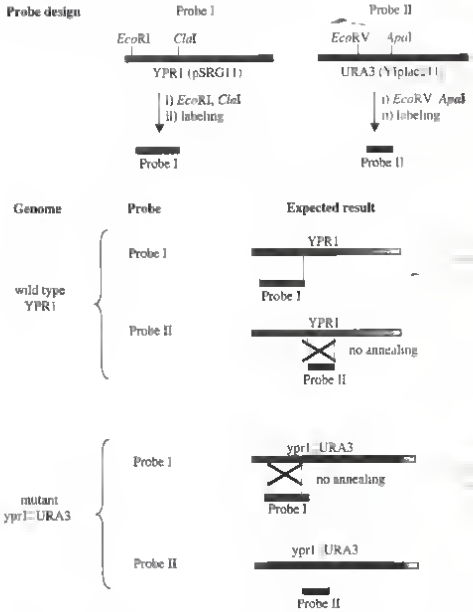
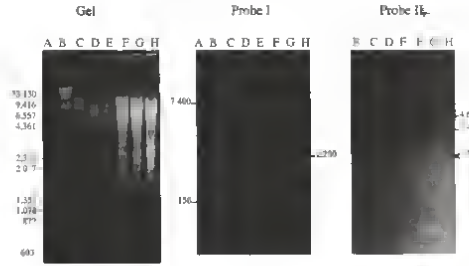


Figure 3-15 Design of the southern blot probes and characterization of the mutant JS3

Three enzymes, *Cla*I, *Eco*RI and *Eco*RV which resulted in different restriction patterns for the mutant and the parental strain were used for the digestion of 15C and JS3

genomic DNA. The digested DNAs were run side by side in an agarose gel blotted onto a nitrocellulose membrane and hybridized with the probes. The results obtained are presented in Figure 3-16 and summarized in Table 3-7

As expected, probe I hybridized only to the parental strain and not to the mutant indicating the absence of the *YPR1* gene in the JS3 mutant strain. The presence of the *URA3* cassette in the *YPR1* loci was confirmed with the second probe. Despite the presence of a small background signal probably associated with the *ura3-Δ52* allele, the results were in agreement with the gene replacement.



A MW marker (ΦX174 DNA-*Hae*III digested)
B MW marker (Lambda DNA-*Hin*DIII digested)
C 15C DNA *Cla*I digested
D 15C DNA *Eco*RV digested
E 15C DNA *Eco*RI digested
F JS3 DNA *Cla*I digested
G - JS3 DNA-*Eco*RV digested
H - JS3 DNA-*Eco*RI digested

Figure 3-16 Southern blot hybridization experiments for characterization of the *ypr1::URA3* knock out in JS3

Table 3-7 Results from the southern blot characterization of the JS2 mutant strain Comparison of expected and actual results for the wild type (15C) and the mutant (JS2) strains

Probe	Digestion enzyme	Expected results		Actual results	
		15C	JS3	15C	JS3
Probe I	*Cla*I	1102	-	1150	
	*Eco*RV	>8000	-	7400	
	*Eco*RI	2170		2200	
Probe II	*Cla*I		5700		4600
	*Eco*RV		3932		3400
	*Eco*RI		2287		2200

Analysis of β-Keto Ester Reductions by Whole Cells of Knock out Mutant Yeast Strains

The mutant strains lacking one of the three reductases fatty acid synthetase, aldo keto reductase or α-acetoxy ketone reductase were tested for whole cell-mediated reductions of β-keto esters and the results were compared with those obtained for the wild type strains. The substrates used in these studies were the same as those previously used for characterization of the over-expression strains (Figure 3-12). Strain 2B lacking fatty acid synthetase, was only tested with substrates 33a-e since the absence of this enzyme would not affect the reduction of α-substituted β keto esters. Biotransformations were performed as described in the previous section and the reaction progress and the enantiomeric and diastereomeric excess values were determined by GC

Reductions using ATCC26403 and 2B, Mutant Strains Lacking Fatty Acid Synthetase Activity

Preliminary results were obtained using ATCC26403. This strain was tested for the reduction of ethyl 3-oxo pentanoate, which yields the (*R*)-enantiomer as the major product when reduced by unmodified baker's yeast. Substitution for ATCC26403 switched the enantioselectivity so that the (*S*)-alcohol was instead produced in 88 % enantiomeric excess. On the other hand, when this substrate was reduced with 2B, which carries a complete *Δfas2* deletion, an even better improvement was obtained with an enantioselectivity greater than 98%. This difference could be related to the different types of mutations or to the different parental strains used to construct each mutant. Given its higher selectivity, the 2B strain was used for the rest of our studies. This strain was used to reduce ketones **33a-e** and the results are summarized in Table 3-8. The absence of fatty acid synthetase in this strain resulted in very high enantioselectivities toward the production of the (*S*)-alcohols. Moreover, the results obtained with substrates **33d** and **e** indicate that fatty acid synthetase may be the only enzyme involved in the reductions of these substrates to yield the (*R*)-enantiomer. That is not the case for substrates **33b** and **c**, however, since production of small amounts of the (*R*)-alcohol was still detected. The effect of the mutation was also noticeable in the biotransformation rate. Reductions involving fas2 knock out strains required 48 h to reach completion whereas the control strain reactions were complete within 24-36 h.

Table 3-8 Effect of the fatty acid synthetase absence on whole cell mediated reductions of β-keto esters

Substrate (33)				Strains	
33	R_1	R_2	R_3	15C	2B
a	Me	H	Me	90 % ee (*S*)	> 98 % ee (*S*)
b	Et	H	Me	70 % ee (*R*)	72 % ee (*S*)[1]
c	Me	H	Et	95 % ee (*S*)	95 % ee (*S*)
d	Et	H	Et	8 % ee (*R*)	98 % ee (*S*)
e	Propyl	H	Et	>98 % ee (*R*)	> 98 % ee (*S*)[1]

[1] Incomplete conversion was observed with these substrates (91 % and 93 % respectively after 48 h)

Biotransformations with JS3, a Strain Lacking Aldo Keto Reductase

When the strain lacking aldo-keto reductase was used to reduce ketones **33a-e**, only a small decrease in the amount of (*S*)-alcohol was observed (Table 3-9). These results indicated that either this enzyme contributes little to the reduction of these substrates in wild-type cells, or its absence is compensated for by the yeast cell. By contrast, the absence of this reductase had a large impact on the diastereoselectivities of reductions of **33f-i** (Table 3-9). As expected, larger amounts of the 2*S* 3*S*-*anti* diastereomers were produced when the mutant strain lacking the aldo-keto reductase was used for reduction of substrates **33f-i**. However, the 2*R* 3*S* *syn* diastereomers were still produced, indicating the presence of other enzyme(s) with the same stereospecificity as aldo keto reductase. Two other yeast reductases with similar diastereoselectivity have been identified (see Chapter 5) and may be associated with these results.

Table 3-9. Comparison of JS3 mutant strain lacking aldo keto [illegible] whole cell-mediated reductions of β keto esters

	Substrate (33)			Strains	
33	R_1	R_2	R_3	ISC	JS3
a	Me	H	Me	90 % ee (*S*)	90 % ee (*S*)
b	Et	H	Me	70 % ee (*R*)	76 % ee (*R*)
c	Me	H	Et	95 % ee (*S*)	93 % ee (*S*)
d	Et	H	Et	8 % ee (*R*)	64 % ee (*R*)
e	Propyl	H	Et	>98 % ee (*R*)	>98 % ee (*R*)
f	Me	Me	Et	>98 % de (*syn*)	74 % de (*syn*)
g	Me	Et	Et	10 % de (*syn*)	30 % de (*anti*)
h	Me	Allyl	Et	59 % de (*anti*)	83 % de (*anti*)
i	Me	Propargyl	Et	30 % de (*syn*)	42 % de (*anti*)

Only partial conversion was observed with these substrates (40 % and 62 respectively after 48 h)

Biotransformations with 10495B, Mutant Strain Lacking α Acetoxy Ketone Reductase

Strain 10495B lacking α acetoxy ketone reductase was tested for the reductions of several β keto esters and the results are presented in Table 3-10. The absence of this enzyme affected the outcomes of whole cell mediated reductions for non-substituted as well as substituted β keto esters. As expected, a significant decrease in the amount of (*S*) alcohol was observed when the mutant strain was used to reduce substrates **33a**, **c** and **d** demonstrating the contribution of this enzyme to these reductions in wild type cells.

Surprisingly the opposite effect was seen when substrates **33b** and **e** were reduced by this knock out strain. For these two ketones production of more (*S*)-enantiomer was detected. This result was reproduced several times and could not be explained simply by the absence of α acetoxy ketone reductase since this enzyme is known to reduce these two ketones to the (*S*)-alcohols. This behavior was confirmed by over-expression of b

[illegible] also observed for the reduction of **33f** which resulted in production of more *anti* diastereomer. The more likely explanation for these outcomes is that the yeast cells counteract the absence of α-acetoxy ketone reductase by over-expressing one or more reductases.

The results obtained for the reductions of substrates **33g** i nicely illustrated the effect of deleting α acetoxy ketone reductase. As expected, larger amounts of the *2S,3R* syn diastereomers were produced when the mutant strain was used for reduction of these substrates. However, the diastereoselectivity was again incomplete. This observation along with the unexpected results obtained for substrates **33h**, **e** and **f**, indicate the presence of other enzymes with similar stereospecificity to α-acetoxy ketone reductase. BLAST searches of the *S. cerevisiae* genome revealed three additional enzymes with very high similarity to α acetoxy ketone reductase that might be responsible for this activity (Chapter 5).

Table 3.10. Characterization of 10495B (mutant strain lacking α acetoxy ketone reductase) for whole cell-mediated reduction of β keto esters.

Substrate (33)				Strains	
33	R_1	R_2	R_3	**15C**	**10495B**
a	Me	H	Me	90 % ee (*S*)	38 % ee (*S*)
b	Et	H	Me	70 % ee (*R*)	50 % ee (*R*)
c	Me	H	Et	95 % ee (*S*)	72 % ee (*S*)
d	Et	H	Et	8 % ee (*R*)	25 % ee (*R*)
e	Propyl	H	Et	>98 % ee (*R*)	67 % ee (*R*)
f	Me	Me	Et	>98 % de (*syn*)	67 % de (*syn*)
g	Me	Et	Et	10 % de (*syn*)	45 % de (*syn*)
h	Me	Allyl	Et	59 % de (*anti*)	12 % de (*anti*)
i	Me	Propargyl	Et	30 % de, (*syn*)	49 % de (*syn*)

Summary of the Improvement on ee/de Obtained with the First Generation of Designed Yeast Strains.

Tables 3-11 and 3-12 summarize the results obtained with the first generation engineered yeast strains. A comparative analysis of the results obtained for different substrates with the three knock out mutants (Table 3-11) along with the results from the over-expression strains (Table 3-12) revealed the role that each reductase might play in β keto ester reductions by baker's yeast. Clearly fatty acid synthetase is the main participant in the formation of alcohols of *R* configuration. Among the enzymes yielding the *S*-alcohol, the larger contribution is made by α acetoxy ketone reductase while aldo keto reductase seems to play a minor role, at least for the ketones investigated here. A similar analysis of the results obtained with α substituted β keto esters **33f-i** showed that both aldo keto reductase and α-acetoxy ketone reductase participate to a large extent in the reduction of these substrates. The effects of over-expressing both enzymes were most clearly seen in the reductions of ethyl α propargyl acetoacetate (**33i**) indicating that both of them present high specific activities for this substrate.

These results have also led to a clearer picture of the β keto esters reductases present in wild type baker's yeast. Clearly the three reductases previously reported in the literature participate in the reductions of β keto esters however other reductases must also be involved. In addition baker's yeast appears to compensate for the loss of at least some ketone reductases.

Table 3.11 Comparative effects on whole cell mediated reductions of β keto esters by mutant strains lacking one of the reductase activities

Substrate (33)				Strain (deleted enzyme)			
33	R_1	R_2	R_3	**2B** (fatty acid synthetase)	**15**[a] (none)	**JS3** (aldo keto reductase)	**E0495B** (α alkoxy ketone reductase)
a	Me	H	Me	> 98 % ee (*S*)	90 % ee (*S*)	90 % ee (*S*)	38 % ee (*S*)
b	Et	H	Me	72 % ee (*S*)	70 % ee (*R*)	76 % ee (*R*)	50 % ee (*R*)
c	Me	H	Et	95 % ee (*S*)	95 % ee (*S*)	93 % ee (*S*)	72 % ee (*S*)
d	Et	H	Et	98 % ee (*S*)	8 % ee (*R*)	14 % ee (*R*)	25 % ee (*R*)
e	Propyl	H	Et	> 98 % ee (*S*)	>98 % ee (*R*)	>98 % ee (*R*)	67 % ee (*R*)
f	Me	Me	Et		>98 % de (*syn*)	74 % de (*syn*)	67 % de (*syn*)
g	Me	Et	Et		10 % de (*syn*)	30 % de (*anti*)	45 % de (*a*
h	Me	Allyl	Et		59 % de (*anti*)	87 % de (*anti*)	12 % de (*a*
i	Me	Propargyl	Et		30 % de (*syn*)	42 % de (*anti*)	49 % de (*s*

Table 3.12 Comparative effects on whole cell mediated reduction of β keto esters by strains over expressing a particular reductase activity

Substrate (33)				Over-expressed enzyme				
33	R_1	R_2	R_3	none (galactose)	fatty acid synthetase	aldo keto reductase	none (glucose)	α acetoxy ketone reductase
a	Me	H	Me	84 % ee (*S*)		93 % ee (*S*)	90 % ee (*S*)	>98 % ee (*S*)
b	Et	H	Me	86 % ee (*R*)		36 % ee (*R*)	70 % ee (*R*)	53 % ee (*S*)
c	Me	H	Et	84 % ee (*S*)		>98 % ee (*S*)	95 % ee (*S*)	>98 % ee (*S*)
d	Et	H	Et	70 % ee (*R*)	88 % ee (*R*)	36 % ee (*R*)	8 % ee (*R*)	81 % ee (*S*)
e	Propyl	H	Et	>98 % ee (*R*)		97 % ee (*R*)	>98 % ee (*R*)	34 % ee (*R*)
f	Me	Me	Et	83 % de (*syn*)		96 % de (*syn*)	>98 % ee (*syn*)	71 % de (*syn*)
g	Me	Et	Et	59 % de (*syn*)		81 % de (*syn*)	10 % de (*syn*)	38 % de (*anti*)
h	Me	Allyl	Et	17% de (*syn*)		65 % de (*syn*)	59 % de (*anti*)	>98 % de (*anti*)
i	Me	Propargyl	Et	28 % de (*syn*)		>98 % de (*syn*)	30 % de (*syn*)	>98 % de (*anti*)

The improvements in the enantio- and diastereoselectivity [illegible] engineered strains indicated that the rational design of yeast strains is [illegible] improving the stereoselectivities of these reductions. In particular, the [illegible] expressing aldo-keto reductase and α-acetoxy ketone reductase provided [illegible] stereoselectivity for some substrates. In the case of ethyl α-propargyl acetoacetate, [illegible] both diastereomers could be prepared in pure optical form, nicely illustrating the potential of the designer yeast approach.

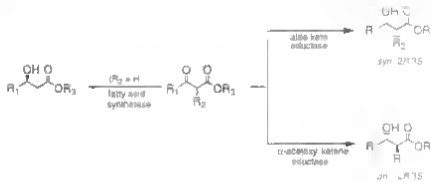
Although the results obtained from this first generation of engineered strains provided good improvements on the stereoselectivity of baker's yeast reductions, incomplete stereoselectivity was still a common outcome of these biotransformations. These results represented new challenges since it indicated that more reductases than those originally thought were involved in the reduction, and that a combination of knock-out and over-expression strategies was necessary to accomplish higher levels of diastereo- and enantioselectivities.

CHAPTER 4
CONSTRUCTION OF A SECOND GENERATION OF ENGINEERED YEAST STRAINS AND THE ASSOCIATED IMPROVEMENTS IN ENANTIO- AND DIASTEREOSELECTIVITY

Despite the good improvements in stereoselectivity associated with the [illegible] generation of "designer yeast" carrying either a knock out or over-expression of a particular yeast reductase, incomplete stereoselectivity was still a common outcome in whole cell-mediated reductions with these strains. By rationally combining knock outs of competing enzymes with the over-expression of the desired activity, we expected to observe further improvements in stereoselectivity. Four new strains with improved stereoselectivity were therefore designed along these lines and an overview of the strategy is summarized in Figure 4-1.

An *S*-enantioselective strain was created by over-expressing α-acetoxy ketone reductase in cells lacking a functional fatty acid synthetase. The opposite enantioselectivity was targeted by combining knock outs of both aldo-keto reductase and α-acetoxy ketone reductase with the over-expression of the fatty acid synthetase α subunit. To generate a "*syn* diastereoselective" strain, aldo-keto reductase was over-expressed in cells lacking α-acetoxy ketone reductase. The opposite combination, over-expression of α-acetoxy ketone reductase in a strain lacking aldo-keto reductase, resulted in a strain with improved *anti* diastereoselectivity.

This second generation of "designer yeast" afforded in most cases better stereoselectivities for whole cell-mediated reductions of β-keto esters than those obtained



Strain	Combined knock out and over-expression	
	deleted enzyme(s)	over-expressed enzyme
S enantioselective (2B(pSRG41))	fatty acid synthetase (α subunit)	α acetoxy ketone reductase
R- enantioselective (24B(pSRG29))	α-acetoxy ketone reductase and aldo keto reductase	fatty acid synthetase (α subunit)
syn diastereoselective (10495B(pSRG14))	α acetoxy ketone reductase	aldo keto reductase
anti diastereoselective (JS3(pSRG41))	aldo keto reductase	α acetoxy ketone reductase

Figure 4-1 Construction of strains with higher stereoselectivity by combination of knock out and over-expression of three of the enzymes involved in β keto ester reduction

Strain Constructions

The construction of the second generation of "designer yeast" strains was done by transforming the appropriate mutant strain carrying the desired deletion with the plasmid for over-expression of the enzyme with opposite stereoselectivity. Transformations were performed according to the high-efficiency lithium acetate protocol[147] and transformants were selected in minimal media with adequate supplements. In this way, transformation of yeast strain 2B with pSRG41 followed by selection in minimal medium lacking leucine and supplemented with fatty acids and geneticin provided the "*S*-enantioselective" strain 2B(pSRG41). Similarly, transformation of strain JS3 with pSRG41 and selection in medium depleted of uracil and leucine afforded the recombinant "*anti*-diastereoselective" strain JS3 (pSRG41). The "*syn*-diastereoselective" strain 10495B (pSRG14) was created by transforming the Euroscarf strain 10495B with pSRG14 and selecting in minimal medium devoid of uracil and supplemented with geneticin. Finally, an "*R* enantioselective" strain lacking both aldo keto reductase and α acetoxy ketone reductase but over-expressing the α-subunit of fatty acid synthetase was constructed. This required the prior generation of the double mutant that was subsequently transformed with pSRG29, and this process is described below.

Construction of a Double Mutant carrying the Deletion of the Aldo Keto Reductase and the α-Acetoxy Ketone Reductase

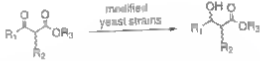
The first step in the construction of the "*R*-enantioselective" strain was the generation of a *Δypr1 Δgre2* double mutant. This mutant was obtained by mating the two single mutants JS3 (*Δypr1*) and 10495B (*Δgre2*), followed by sporulation of the diploid strain and screening for the desired genotype. The new strain was then transformed with

pSRG29 (which directs [illegible] fatty acid synthetase [illegible]) provide the *R*-enantioselective strain 24B(pSRG29)

The diploid strain was generated by mating the parental strains JS[illegible]-2498B in minimal solid medium depleted of both uracil and histidine. Once cells of the diploid strain developed they were transferred to pre sporulation medium and then to sporulation media to induce spore formation. The spores were dissected and screened for colonies able to grow without uracil and in the presence of geneticin. A total of 29 tetraspores were dissected and plated in YPD. The master plate containing the spore progeny was replica plated on two different selective media for screening, one containing geneticin and the second one depleted of uracil. The spores carrying the gre2::kanMX4 gene replacement were able to grow in presence of geneticin, and those carrying the ypr1::URA3 mutation were able to grow in the absence of uracil. The screening indicated the presence of 5 parental ditype, 7 non-parental ditype, and 17 tetratype tetrads. Three colonies that were able to grow in both media, indicating presence of the double mutation, were further screened for altered stereoselectivity in β keto ester reductions. Two of the three spores gave the expected phenotype: a decreased amount of S alcohol from whole cell mediated reductions of β keto esters (*vide infra*). One of these strains (24B) was selected for further constructions and characterization. The plasmid over expressing the α subunit of fatty acid synthetase (pSRG29) was introduced into 24B by the lithium acetate method [47].

Analysis of Whole Cell Mediated Reductions of β-Keto Esters by the C[illegible] Yeast Strains

The four second-generation "designer yeast" strains were tested for whole cell mediated reductions of β keto esters and the results were compared with those obtained from both the unmodified strain and the first generation of engineered strains. The substrates employed for these studies were the same as those used previously (Figure 4-2). Substrates 33a-e were used to test the improvements in enantioselectivity provided by the strains 24B(pSRG29) and 28D(pSRG41) while substrates 33f-e helped characterize the improvements in diastereoselectivity associated with strains JS3(pSRG41) and 10495B(pSRG14).



33 34

a R_1 = Me R_2 = H R_3 = Me
b R_1 = Et R_2 = H R_3 = Me
c R_1 = Me R_2 = H R_3 = Et
d R_1 = Et, R_2 = H R_3 = Et
e R_1 = n-Pr R_2 = H R_3 = Et

f R_1 = Me R_2 = Me R_3 = Et
g R_1 = Me R_2 = Et, R_3 = Et
h R_1 = Me R_2 = allyl R_3 = Et
R_1 = Me R_2 = propargyl, R_3 = E

Figure 4-2. Substrates used for testing the improvements in stereoselectivity associated with the second generation of "designer yeast" strains.

Biotransformations were performed as described in the previous chapter. The reaction progress was monitored by GC and the enantiomeric and diastereomeric excess values were determined by chiral-phase GC. The reactions were performed on scales sufficient for product isolation and characterization by NMR and optical rotation.

Effects Associated with the Combined Deletion of α Acetoxy Ketone Reductase and Aldo Keto Reductase, along with the Over Expression of Fatty Acid Synthetase

Strain 24B defective in both aldo keto reductase and α acetoxy ketone reductase was first analyzed for the effect of the double mutation on the enantioselectivity of reductions. Two substrates **33c** and **33d** were used in this initial characterization and the results were compared with the improvements provided by each single mutation (Table 4-1). Despite the low effect that each individual gene knock out had on the outcome of these reductions, the double mutant showed dramatic changes. Attempted reduction of ethyl acetoacetate (**33c**) resulted in almost no conversion, while ethyl 3-oxopentanoate (**33d**) was reduced to produce more *R*-enantiomer than was observed for either single mutant. Identical results were obtained with strains derived from two spores, 24B and 16C, both of which were shown to carry the double mutation from the previous experiments. This conclusively established that the results were indeed associated with the *Δypr1 Δgre2* genotype.

Table 4-1. Effect of the double mutation *Δgre2- Δypr1* on the enantioselectivity of β-keto ester reductions (using glucose as carbon source)

Substrate (33)			Effect on the enantioselectivity			
R_1	R_2	R_3	control	knock out of Gre2p	knock out of Ypr1p	double mutant
Me	H	Et	95 % ee (*S*)	72 % ee (*S*)	93 % ee (*S*)	almost no conversion
Et	H	Et	8 % ee (*R*)	25 % ee (*R*)	14 % ee (*R*)	60 % ee (*R*) incomplete conversion

Since the expression system for fatty acid synthetase requires [illegible] carbon source and the presence of this sugar has been shown to favor [illegible] enantiomer (Chapter 3), the control experiments for the double mutant 24B[illegible] in media containing galactose (Table 4-2). Although ethyl acetoacetate was [illegible] these experiments only 50 % of the substrate was consumed after 46 h. As in the previous experiments, it was clear that the double knock out affected the reduction of β-keto esters to a larger extent than would have been anticipated from either single mutant.

Table 4-2. Effect of the double mutation *Δgre2 Δypr1* on the enantioselectivity of β-keto ester reductions [using galactose as carbon source].

Substrate (33)			Effect on the enantioselectivity			
R_1	R_2	R_3	control	knock out of Gre2p	knock out of Ypr1p	double mutant
Me	H	Et	84 % ee (*S*)	53 % ee (*S*)	82 % ee (*S*)	>90 % ee (*S*), no significant conversion
Et	H	Et	70 % ee (*R*)	88 % ee (*R*)	78 % ee (*R*)	98 % ee (*R*), incomplete conversion

Incomplete conversion was also observed in reactions with 24B/pSRG2[illegible] which over-expresses the α subunit of fatty acid synthetase but lacks both aldo keto reductase and diacetoxy ketone reductase (Table 4-3). The low conversion was reflected in lower yields than those usually obtained from baker's yeast reductions. Despite this drawback some improvements in the enantioselectivity were observed with the new strain.

A comparison of the results obtained with the single mutants and the double mutant over-expressing the α subunit of fatty acid synthetase is presented in Table 4-4.

The single mutant 10495B carrying the deletion of α acetoxy ketone reductase (knock out) resulted in a larger production of the *R*-enantiomer for substrates **33a** and **33h**. Contrary to what was expected, the combination of this knock out with the diketo keto reductase (Ypr1p) knock out and with fatty acid synthetase (Fas2p) over-expression actually resulted in lower improvements. The Ypr1p knock out alone did not contribute much to formation of the *R*-alcohol but it was not expected that it would result in the opposite contribution (entry 1). A possible reason for this outcome relates to the metabolism of strains. The double mutant is a combination of both single mutants and therefore differ in background reductase activity from either single mutant.

The second generation strain provided slightly better results than the single mutants for the biotransformations of substrates **33e** and **33d** (Table 4-4). These results are more likely related to the combination of knock outs than to the over-expression of fatty acid synthetase, since this over expression did not have a major impact and presented problems of plasmid retention [3]. From the results presented in Table 4-2, it appeared as if the double mutant 24B provided better enantioselectivity than the recombinant strain 24BtpSRG29; however, biotransformations with the latter were allowed to proceed for longer times to achieve greater conversion and the difference in enantioselectivity may be merely associated to this variable. The relative concentration of the different reductases within the cells changes over time and the newly formed product would reflect those changes affecting the overall stereoselectivity of the reaction.

[3] Only 16 % of the cells retaining the plasmid after 24 h and complete lost of the plasmid was observed after 50 h of growth in non selective media (the time required for the biotransformation).

Unfortunately the overall improvement obtained with the second generation strain was not commensurate with the complicated genetics of the strain. Similar improvements and better yields were obtained with the first generation strain 10495B defective in α-acetoxy ketone reductase. Further improvements towards synthesis of the *R* alcohols might be achieved by over-expressing a D enzyme other than fatty acid synthetase in strain 10495B. This possibility was not explored further.

Table 4-3 Results from large scale whole cell mediated reduction of substrates **33a-e** with strain 24B(pSRG29)

Entry	Substrate (33)			Biotransformation with 24B(pSRG29)				
	R_1	R_2	R_3	conversion (after 60 h)	isolated yield	% ee	abs. conf	$[\alpha]_D^{25}$ (c, $CHCl_3$)
1	Me	H	Me	53 %	28 %	72	*S*	+ 31.44 (c 0.7)
2	Et	H	Me	86 %	80 %	88	*R*	12.5 (c 2.0)
3	Me	H	Et	79 %	66 %	64	*S*	+ 15.8 (c 2.5)
4	Et	H	Et	>98 %	78 %	91	*R*	21.8 (c 2.5)
5	Propyl	H	Et	40 %	26 %	>98	*R*	15.8 (c 2.5)

Table 4-4 Effect of combining the knock out of aldo keto reductase and α acetoxy ketone reductase with the over expression of Fas2p

Entry	Substrate (33)			Effect on the enantioselectivity			
	R_1	R_2	R_3	control	knock out of Gre2p	knock out of Ypr1p	combined strain
1	Me	H	Me	84 % ee (*S*)	93 % ee (*S*)	85 % ee (*S*)	72 % ee (*S*)
2	Et	H	Me	86 % ee (*R*)	95 % ee (*R*)	86 % ee (*R*)	88 % ee (*R*)
3	Me	H	Et	84 % ee (*S*)	68 % ee (*S*)	82 % ee (*S*)	64 % ee (*S*)
4	Et	H	Et	79 % ee (*R*)	88 % ee (*R*)	78 % ee (*R*)	91 % ee (*R*)
5	Propyl	H	Et	>98 % ee (*R*)	>98 % ee (*R*)	>98 % ee (*R*)	>98 % ee (*R*)

Stereoselectivity of Strain 2BrpSRG41), Carrying a Deletion of Fatty Acid Synthetase, and Over Expressing α Acetoxy Ketone Reductase

The results obtained with this strain provide a clear example of the benefits of combining the over-expression of the desired enzyme with the knock out of a major competing enzyme. Biotransformations with this strain were completed in less than 24 h with very good yields and provided the optically pure *S* alcohol for all the substrates tested (Table 4-5).

For substrates **33a** and **33c**, complete enantioselectivity had been already achieved by over-expressing α acetoxy ketone reductase (Gre2p); however, for the other substrates, the over-expression of this enzyme was not sufficient to completely shift the enantioselectivity (Table 4-6). However, by combining the fatty acid synthetase Fas2p knock out with the over-expression of α acetoxy ketone reductase, the reductions of substrates **33b**, **d** and **e** gave the optically pure *S* alcohols. Unmodified baker's yeast provides the *R* alcohol as the major product for these reductions (Table 4-6). The most noticeable example was the reduction of **33e**, which changed from >98% ee *R* alcohol with the control strain to >98% ee *S* alcohol with the second generation engineered strain.

Table 4.5 Results from large scale whole cell mediated reduction of substrate 33a-e with strain 2B(pSRG41)

Substrate (33)			Biotransformation with 2B(pSRG41)				
R_3	R_2	R_1	conversion	isolated yield	% ee	abs. conf.	$[\alpha]_D^{25}$ (c, $CHCl_3$)
Me	H	Me	>98 %	76 %	>98	S	+ 40.1 (c 2.5)
Et	H	Me	>98 %	85 %	>98	S	+ 29.4 (c 2.5)
Me	H	Et	>98 %	83 %	>98	S	+ 32.5 (c 2.5)
Et	H	Et	>98 %	87 %	>98	S	+ 26.2 (c 2.5)
Propyl	H	Et	>98 %	90 %	>98	S	+ 16.5 (c 2.5)

Table 4.6 Effect of combining the fatty acid synthetase knock out with the R-ketoxy ketone reductase over expression

Substrate (33)			Effect on the enantioselectivity			
R_3	R_2	R_1	control	knock out of FasZp	over-expression of Grx2p	combined strain
Me	H	Me	90 % ee (S)	> 98 % ee (S)	>98 % ee (S)	>98 % ee (S)
Et	H	Me	70 % ee (S)	72 % ee (S)	>98 % ee (S)	>98 % ee (S)
Me	H	Et	85 % ee (S)	91 % ee (S)	>98 % ee (S)	>98 % ee (S)
Et	H	Et	>98 % ee (S)	>98 % ee (S)	>98 % ee (S)	>98 % ee (S)
Propyl	H	Et	>98 % ee (S)	>98 % ee (S)	>98 % ee (S)	>98 % ee (S)

Stereoselectivity of Strain 10495B(pSRG14), carrying a Deletion of α-Acetoxy Ketone Reductase and Over Expressing Aldo Keto Reductase

Strain 10495B(pSRG14) lacking α acetoxy ketone reductase and over expressing aldo keto reductase provided the *syn* (2*R* 3*S*)-**34** alcohols in good yields and good stereoselectivities (Table 4-7). For substrates **33f** and **33i** only single diastereomers were observed after completion of the reaction.

When compared to the results obtained from a strain over expressing aldo keto reductase (Ypr1p), the second generation strain provided slight improvements in the diastereoselectivity for substrates **33f** and **33g**, and no improvement for substrate, **33h** (Table 4-8). A larger influence of the α acetoxy ketone reductase (Gre2p) knock out was expected since it provided good improvements in diastereoselectivity when a strain carrying only this deletion was analyzed (Chapter 3). However, previous studies were performed with glucose as the carbon source, while the over-expression of aldo keto reductase instead required the use of galactose. Based on our previous studies, the effect of the α acetoxy ketone reductase knock out was much lower in galactose as compared to reductions with glucose as the carbon source. Another factor that could also contribute to the low additional increase in diastereoselectivity observed for the second generation strain is the difference in parental strains, which may result in different levels of protein over-expression from the pSRG14 vector. The first-generation recombinant strain was based on 15C, while 10495B was used for the second strain.

Table 4.7 Results from large-scale whole cell-mediated reduction of substrates **33** by wild strain 10495B/pSRG [4]

Substrate (33)			Bioransformation with 10495B/pSRG [4]				
R_1	R_2	R_3	conversion	isolated yield	%ee, %de	abs. conf.	$[\alpha]_D^{25}$ (c, $CHCl_3$)
Me	Me	Et	> 98 %	90 %	> 98, 98	(2*R*,3*S*)	+ 3.68 (c, 2.5)
Me	Et	Et	> 98 %	89 %	> 98, 83	(2*R*,3*S*)	+ 9.32 (c, [illegible])
Me	Allyl	Et	> 98 %	92 %	> 98, 65	(2*R*,3*S*)	+ 6.36 (c, [illegible])
Me	Propargyl	Et	> 98 %	75 %	> 98, >98	(2*R*,3*S*)	+ 14.8 (c, [illegible])

Table 4.8 Effect of combining the α-acetoxy ketone reductase knock out with the aldo-keto reductase over-expression

Substrate (33)			Effect on the stereoselectivity			
R_1	R_2	R_3	control	knock out of Gre2p	over-expression of Ygr1p	combined strain
Me	Me	Et	83 % de (anti)	98 % de (anti)	96 % de (anti)	98 % de (anti)
Me	Et	Et	59 % de (anti)	64 % de (anti)	81 % de (anti)	83 % de (anti)
Me	Allyl	Et	17 % de (anti)	28 % de (anti)	60 % de (anti)	[illegible]
Me	Propargyl	Et	28 % de (anti)	95 % de (anti)	> 98 % de (anti)	[illegible]

Stereoselectivity of Strain JS3 (pSRG41), carrying a Deletion of Aldo keto Reductase and Over Expressing α Acetoxy Ketone Reductase

This strain reduced substrates **33g i** to provide the *anti* (2*S*,3*S*) **34** alcohols in good yields and good stereoselectivities (Table 4.9). For substrates **33h** and **33i** the above-mentioned alcohol was the only product observed after completion of the reaction. On the other hand, reduction of **33f** afforded the *syn* (2*R*,3*S*) alcohol as the major product. This was not surprising since all the enzymes whose concentrations were altered in this strain reduce this substrate to the *syn* diastereomer.[60,108]

A comparison of the results obtained with the second generation strain and those obtained with the single mutant or over expression is presented in Table 4.10. The over expression of α-acetoxy ketone reductase (Gre2p) already provided complete diastereoselectivity for the reduction of substrates **33h** and **33i**, thus the contribution of the aldo keto reductase (Ypr1p) knock out was hidden in these results. However, reduction of **33g** with the first generation strains resulted in formation of the *anti* (2*S*,3*S*) alcohol with poor diastereoselectivity. The combination of the aldo keto reductase knock out and α acetoxy ketone reductase over-expression resulted in additive effects providing the desired alcohol with better diastereoselectivity. Unfortunately, incomplete stereoselectivity was still seen for **33g**, and more efforts may be necessary to achieve complete diastereoselectivity for this substrate.

Table 4.9 Results from large scale whole cell mediated reduction of substrates (33) with strain JS3(pSRG411)

Substrate (33)			Biotransformation with JS3(pSRG411)				
R_1	R_2	R_3	conversion	isolated yield	%ee, %de	abs. conf.	$[\alpha]_D^{25}$ (c, CHCl$_3$)
Me	Me	Et	> 98 %	86 %	> 98, 70	(2*R*,3*S*)	+ 8.04 (c, 2.51
Me	Et	Et	> 98 %	73 %	> 98, 67	(2*S*,3*S*)	+ 5.56 (c, 3.[illegible]
Me	Allyl	Et	> 98 %	67 %	> 98, >98	(2*S*,3*S*)	+ 15.04 (c, 3.[illegible]
Me	Propargyl	Et	> 98 %	70 %	> 98, >98	(2*S*,3*S*)	+ 5.16 (c, 2.51

Table 4.10 Effect of combining the aldo keto reductase knock out with the reductive ketone reductase over expression

Substrate (33)			Effect on the stereoselectivity			
R_1	R_2	R_3	control	knock out of Ypr1p	over-expression of Grc2p	combined strain
Me	Me	Et	>98 % de (syn)	74 % de (syn)[1]	71 % de (syn)	70 % de (syn)
Me	Et	Et	70 % de (syn)	80 % de (syn)[1]	88 % de (syn)	67 % de (syn)
Me	Allyl	Et	89 % de (syn)	85 % de (syn)	>98 % de (syn)	98 % de (syn)
Me	Propargyl	Et	80 % de (syn)	42 % de (syn)	>98 % de (syn)	>98 % de (syn)

Summary of the Results Obtained with the Second Generation of "Designer Yeast" Strains and Comparison with Previous Improvements on Baker's Yeast Stereoselectivity

The second generation of "designer yeast" strains provided good improvements in the stereoselectivity of whole cell-mediated reductions of β keto esters. As expected, the combination of gene knock out and over-expression generally provided better results than those obtained from the first generation of yeast strains. Among the improvements in baker's yeast stereoselectivity previously reported in the literature, the use of inhibitors provided the best improvements for some substrates while for others better improvements were obtained by the use of organic co-solvents or changes in the growing conditions (Tables 1-6, 1-9). Comparisons of the best improvements reported in the literature with those obtained along this work for substrates **33a-i** are summarized in Figures 4-3 to 4-6. The analysis is divided into four charts that focus on improvements towards production of the *R*-alcohol, the *S* alcohol, the *syn* diastereomer and the *anti* diastereomer. For convenience, the enantiomeric excess has been represented as positive for the *S* enantiomer and negative when the *R* alcohol was the major product. Similarly, the *syn* diastereomer has been represented as positive while a negative sign indicates an excess of the *anti* diastereomer.

The improvements in stereoselectivity provided by the "*R*-enantioselective" strain 24B(pSRG29) were usually slightly lower than those obtained by other methodologies with the exception of substrate **33a**, for which no improvements in the production of the *R* alcohol have been reported (Figure 4-3). The addition of allyl alcohol provided the best improvements for substrates **33b** and **33c** [121] while limited oxygen availability resulted in 96% ee (*R*)-alcohol from the reduction of **33d** [120] and the presence of DMSO, glucose in

[illegible] provided **34e** >98% ee (*R*). Despite the better [illegible] these approaches. It is clear that they do not represent a [illegible] conditions for good enantioselectivity have to be optimized for each [illegible]. In contrast the "designed yeast" strategy provides a methodology that can be [illegible] the substrates, however, further improvement of this strain is needed to accomplish synthesis of the *R* alcohols with good enantioselectivities. The identification and expression of a D-selective enzyme easier to express than fatty acid synthetase may provide better results.

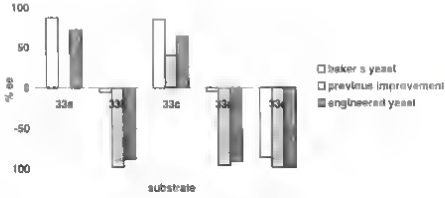


Figure 4-3 Improvements provided by the engineered yeast strain towards the production of the *R* alcohol -comparison with previous reports

The potential associated with the "designed yeast" approach is nicely illustrated by the results obtained with the "*S*-enantioselective" strain 2B(pSRG411). All the substrates tested were reduced by this strain to furnish the *S*-alcohol with >98% optical

[illegible] For substrates **33a-d** these [illegible] slightly higher [illegible] obtained with baker's yeast in the presence [illegible] reductase as an [illegible] 4:4)[97] and the yields are higher with the engineered yeast. The most outst[illegible] obtained with the engineered yeast approach is associated with the reduction of **33e**. The best results previously reported for production of *S*-**34e** resulted in only 2 % ee,[98] while the new strain 2β(pSRG41) provided this β-hydroxy ester in >98 % ee.

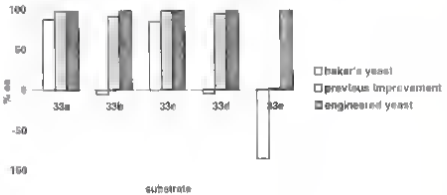


Figure 4-4 Improvements provided by the engineered yeast strain towards the production of the *S* alcohol: comparison with previous reports.

The improvements towards the diastereoselective synthesis of *syn*-(2*R*,3*S*)-2-substituted β-hydroxy esters provided by the strain 10495B(pSRG14) were, with one exception, slightly higher or comparable with those reported previously (Figure 4-5).[99] High diastereoselectivity was previously induced by a combination of heat treatment and addition of the enzyme inhibitor methyl vinyl ketone (MVK) (Table 4-8).[100] For two of

the substrates **33f** and **33i** reduction with the engineered strain provided the best results and the *syn* diastereomer was furnished in >98% optical purity However for substrate **33b** the previously reported strategy provided better diastereoselectivity For substrate **33g** the results were comparable Unfortunately the use of heat and MVK required extreme conditions for good diastereoselectivity and the reduction of 1mM substrate required 20 g of yeast cells and the addition of 60 mM MVK The need for a large mass of yeast cell is not surprising since such a large concentration of MVK nearly inhibits the desired enzyme activity Biotransformations under such conditions resulted in lower yields than those obtained with the engineered yeast Considering these factors along with the analysis of the improvements in diastereoselectivity the engineered yeast provides for most substrates a better choice for the synthesis of *syn*-(2*R* 3*S*)-α substituted β hydroxy esters than any previously reported biocatalytic route

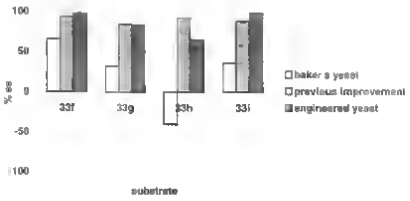


Figure 4-5 Improvements provided by the engineered yeast strain towards the production of the *syn*-diastereomer comparison with previous reports

Improvements towards formation of the *anti*-(2*S* 3*S*) diastereomer in baker's yeast-mediated reductions of α-substituted β keto esters have not been previously reported. The engineered yeast JS3(pSRG411) provided access to optically pure *anti*-(2*S* 3*S*)-**34h** and **34i** that could only be obtained before with purified β keto ester reductases (Table 1-4).[60,108] On the other hand, an excess of the *anti* diastereomer could not be obtained for the reduction of **33f** and reasons for this have been discussed above.

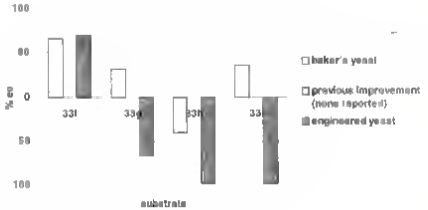


Figure 4-6. Improvements provided by the engineered yeast strain towards the production of the *anti*-diastereomer: comparison with previous reports.

In summary, the engineered yeast strains almost always resulted in comparable or better improvements in stereoselectivity than previously reported methodologies. It provides a general strategy, uses a low cell mass and does not require any co-substrates or enzyme purification. These advantages can make the engineered yeast a more useful reagent for large scale reactions.

CHAPTER 5
IDENTIFICATION OF OTHER REDUCTASES IN BAKER'S YEAST AND ANALYSIS OF THEIR SUBSTRATE SPECIFICITY

Among the reductase enzymes present in baker's yeast aldo keto reductase (Ypr1p) and α acetoxy ketone reductase (Gre2p) had been suggested to be the key participants in production of *syn* (2*R*,3*S*) and *anti* (2*S*,3*S*) α-substituted-β hydroxy esters respectively [80,108] However the results presented in Chapter 3 indicated that although these two enzymes are involved in such reductions, they are not the only ones involved Further work in the rational design of yeast strains with defined stereoselectivities requires that all the relevant proteins participating in each reduction be known in addition to their corresponding genes Although several reductases have been isolated from yeast cells and their substrate specificities have been studied (Table 1-4) there are few cases in which the participating enzyme and the gene encoding for it have been identified [15 107 110 137] Classical genetics has revealed the connection between gene and protein in several cases including yeast alcohol dehydrogenases and fatty acid synthetase [94] In cases where even a fragment of the amino acid sequence data from a purified reductase is available, the gene assignment is unequivocal This method was used to show that the *GRE2* gene encodes α-acetoxy ketone reductase and *YPR1* encodes a yeast aldo keto reductase The major disadvantage of this approach is the time and effort required for isolating each protein and determining its amino acid sequence

The availability of the *S. cerevisiae* genome sequence[136] allows a new approach to the problem of identifying all potential ketone reductases by detecting yeast proteins with sequence similarity to known reductases. During the course of this project the first systematic analysis of the yeast genome for potential reductases has been recently reported by Stewart et al.[104] and this study identified close to 50 possible candidates encompassing members of the short and medium alcohol dehydrogenase aldose reductase and D-hydroxyacid dehydrogenase families lactate dehydrogenases and fatty acid synthetase. The results of this analysis and our earlier experience made it clear that many more reductases than those originally considered in this project may be involved in baker's yeast reduction of ketones.

Yeast Proteins Similar to α-Acetoxy Ketone Reductase (Gre2p)

Analysis of the *S. cerevisiae* genome indicated the presence of three open reading frames with high homology with Gre2p (Figure 5-1). The nature of residues conserved between these open reading frames, the Gre2p and plant cynnamoyl-CoA reductase argues strongly that the proteins encoded by *YDR541c*, *YGL039w* and *YGL157w* are all likely to be functional reductases. All these proteins share the consensus motif Y X X X K that places them in the short-chain alcohol dehydrogenase superfamily.[148]

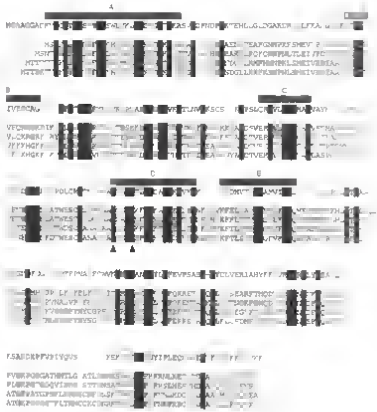


Figure 5.1 Sequence alignment between cider tree cinnamoyl-CoA reductase Gte2p and the protein products of YDR541c YGL039w and YGL157w. Residues conserved across all five sequences are shown against a black background while those positions occupied by two closely related amino acids are shown with a grey background. Sequence blocks labelled with letter correspond to the conserved regions of short-chain alcohol dehydrogenases defined by Krozowski [145]. The location of the Y – X – X – X – K motif is indicated by two black triangles. Taken from 'Cloning, Structure and Activity of Ketone Reductases from Baker's Yeast' in 'Enzyme Technology for Pharmaceutical and Biotechnological Applications' [104].

Yeast Proteins Similar to Aldo Keto Reductase (Ypr1p)

The use of the Ypr1p sequence as probe for BLAST search of the *S. cerevisiae* genome uncovered five additional proteins that were closely related to one another and to human aldose reductase (Figure 5.2). It is very likely that these additional five proteins are also ketone reductases, and they may participate in the reduction of β-keto esters. Of these five, those encoded by the *GCY1*, *ARA1* and *GRE3* genes possessed higher similarity to Ypr1p and were therefore investigated further. Gcy1p has been previously isolated and shown to have high homology with representatives from the three subgroups of the aldo keto reductase family.[133] Gre3p was reported as the aldo-keto reductase responsible for conversion of xylose to xylitol in *S. cerevisiae*.[134] This enzyme was shown to reduce several aldehydes with high catalytic efficiency and *p*-nitro benzaldehyde was the best substrate of those analyzed. Recent research related to the oxidative metabolism of D-arabinose identified a heterodimeric arabinose dehydrogenase in yeast,[135] whose heavy subunit (Ara1p) showed close homology to the aldo-keto reductase family. This was the first report on a heterodimeric protein of the aldo keto reductase family.

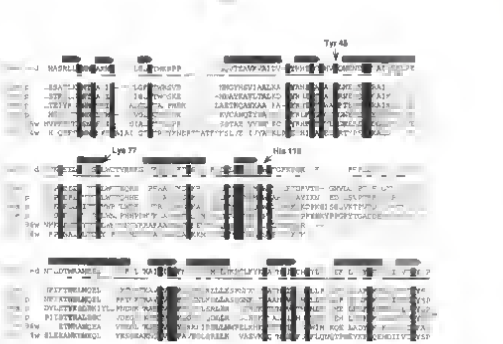


Figure 5 2. Sequence alignment between human aldose reductase (Ald red) and six yeast proteins Ypr1p, Gcy1p, Ara1p, Gre3p, Yjr096w and Ydl124w. Conserved residues are colored as in Figure 5 1. Grey arrows above the mark the positions of β strands and grey bars represent α helices from the X ray crystal structure of this protein (2ACS 4 t. Residues highly conserved between vertebrate aldose reductases are indicated by heavy lines beneath the sequence of human aldose reductase. Taken from "Cloning, Structure and Activity of Ketose Reductases from Baker's Yeast" in Enzyme Technology for Pharmaceutical and Biotechnological Applications" 104

Although each of these three proteins –Gcy1p, Gre3p and Ara1p- share high amino acid sequence identity with Ypr1p, their role in reducing β keto ester had not been investigated. In order to learn whether these enzymes were involved on β-keto ester reductions, expression systems for each of them were designed and tested in baker's yeast and *E. coli*. The expression of these enzymes in both microorganisms provided greater opportunities for studying their activities. As it was previously seen, tight control of protein expression in yeast might hide the effect of over-expressing a reductase (Chapter 3). On the contrary, the use of *E. coli* as a host avoided that problem. In addition, the low level of reductase activity present in *E. coli* furnished a good background to study enzyme activity in whole cell systems. Despite these advantages, expression of an eukaryotic protein in *E. coli* can present problems if post translational modification is essential for activity.

Over-Expression of Gcy1p, Gre3p and Ara1p in Yeast and the Effect on the Stereoselectivity of β-Keto Ester Reductions

Expression systems based on the *S. cerevisiae* expression vector pYES2 were constructed for Gcy1p, Gre3p and Ara1p by standard methods (Figure5-3, 5-4). The three genes were amplified from *S. cerevisiae* genomic DNA using primers that incorporated suitable restriction sites for the cloning strategy. After amplification and restriction enzyme digestion, the *GRE3* gene was cloned directly into pYES2 as an *Eco*RI-*Xba*I cassette, resulting in the expression vector pSRG26 (Figure 5-3). A similar strategy was used to clone *GCY1* between the *Eco*RI and *Xba*I sites of pYES2, providing pSRG28 (Figure 5-3). Direct cloning of the *ARA1* gene was unsuccessful, and a two step strategy was therefore necessary. The *ARA1* PCR product was first cloned into pCR 2.1 and then

subcloned in pYES2 as a *Bam*HI, *Not*I cassette, resulting in pSRG35 (Figure 5-4). In all these vectors, gene expression is under control of the GAL promoter, so that growing the yeast cells in presence of galactose induces enzyme production.

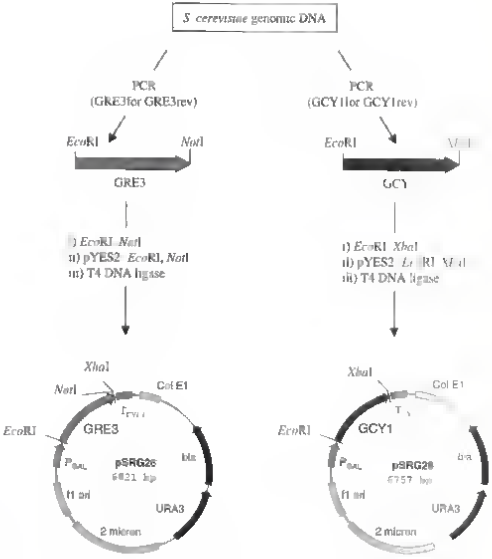


Figure 5.3 Construction of yeast over-expression systems for Gcy1p and Gre3p

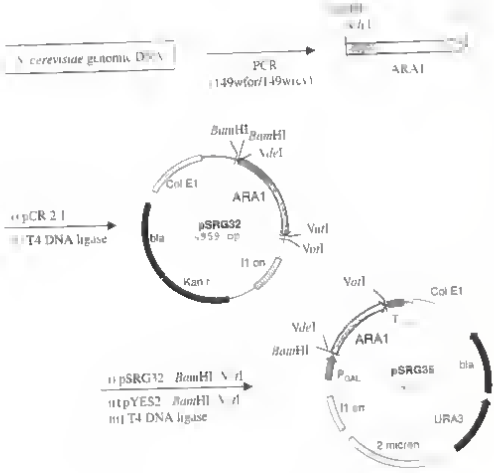


Figure 5-4 Construction of a yeast over-expression system for Ara1p

Each of the three expression plasmids were used to transform *S. cerevisiae* strain 15C and the resulting recombinant strains were tested for changes in the stereoselectivity of β-keto ester reductions with some model substrates (Figure 5-5 Table 5-1). The result indicated that the stereoselectivity of the reduction was unaffected by the over-expression of these enzymes in baker's yeast

R_1 = CH_3 or CH_2CH_3

R_2 = H or allyl

R_3 = CH_2CH_3

Figure 5.1 Model substrates used for testing the effect of Gcy1p Gre3p and [illegible] expression on the stereoselectivity of whole cell yeast-mediated β keto ester [illegible]

Table 5.1 Effects of Gcy1p Gre3p and Ara1p over expression in the stereo[illegible] whole cell yeast-mediated β keto ester reductions

Substrate			Enzyme being over-expressed			
R_1	R_2	R_3	none	Gcy1p	Gre3p	Ara1p
CH_2CH_3	H	CH_2CH_3	70 % ee (*R*)	67 % ee (*R*)	64 % ee (*R*)	[illegible]
CH_3	allyl	CH_2CH_3	17 % de (syn)	21 % de (syn)	13 % de (syn)	[illegible]

Previous reports on the substrate specificity of isolated Ypr1p with several compounds indicated that ethyl 4-chloro-acetoacetate was the best substrate for the enzyme [1]. Attempts to reduce this substrate with growing cells of recombinant [illegible] were unsuccessful due to its high toxicity. Based on this finding, a new assay for detecting additional β-keto ester reductase activity in recombinant yeast was developed. Reduction of ethyl 4-chloro-acetoacetate would dramatically reduce its activity as an alkylating agent, and the strains over-expressing a ketone reductase activity would therefore be less susceptible to this compound. Studies with the yeast strain over expressing Ypr1p indicated that this was indeed the case. Inhibition zone experiments showed that this strain overcame the toxicity reducing by half the radius of inhibition

caused by ethyl 4-chloro-acetoacetate (table [illegible] used to test the strains over-expressing Gcy1p, Gre3p and Ara1p. [illegible] none of them overcame the toxicity of ethyl 4-chloro acetoacetate to a significant [illegible] although they showed a slight decrease in the radius of inhibition (table 5.2).

Table 5.2. Study of ethyl 4-chloro acetoacetate inhibition zone for the mutant [illegible] over expressing Gcy1p, Gre3p and Ara1p.

Over-expressed enzyme	none	Ypr1p	Gcy1p	Gre3p	[illegible]
Inhibition zone radius (cm, 40 h)	3.5	2.7	3.4	3.3	
Inhibition zone radius (cm, 5 days)	3.45	1.61	3.25	3.0	1.[illegible]

Taken together, these results strongly argue that Gcy1p, Gre3p and Ara1p [illegible] play a major role in whole cell reductions of β keto esters. If that is the case, the incomplete stereoselectivity observed after the knock out of the *YPR1* gene must be due to the activity of a different enzyme. Two other members of the aldo-keto reductase family whose sequences are related to that of Ypr1p (hypothetical proteins encoded by *YJR096w* and *YDL124w*) remain candidates. It is also possible that the observed residual activity may be associated with a protein belonging to a different family. However, a second possibility should also be considered: the absence of changes in the stereoselectivity associated with the over-expression of Gcy1p, Gre3p and Ara1p in yeast cells may be due to a tight control of their expression level in baker's yeast.

Expression of Gcy1p, Gre3p and Ara1p in *E. coli*. Study of their Substrate Specificity

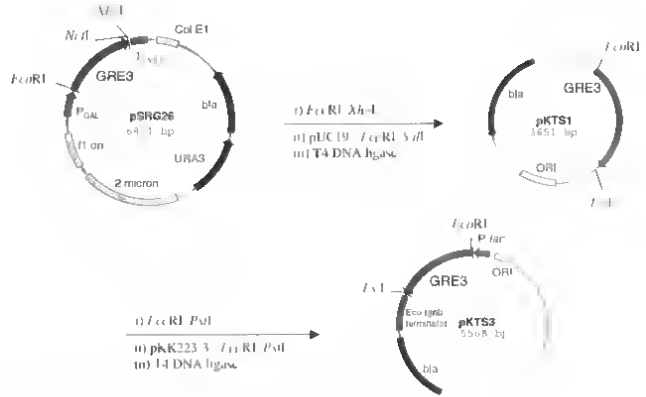
Although the results from overexpressing Gcy1p, Gre3p and Ara1p in baker's yeast seemed to indicate that they were not involved significantly in β-keto ester reductions, their kinship with Ypr1p strongly argued for their participation in the reduction of some type of carbonyl compounds. In order to study the substrate specificity of these reductases, *E. coli* expression systems were made and recombinant strains expressing Gcy1p, Gre3p and Ara1p were constructed. Expressing the enzymes in a different host avoided the control of expression levels that might be present in yeast. *E. coli* provides an ideal microorganism for expression of these yeast enzymes since the level of intrinsic reductase(s) is low. Unmodified *E. coli* has been shown to reduce ethyl 2-methyl acetoacetate[64] and our results indicated that it could also reduce several other β-keto esters to a small degree. Fortunately, this background activity is so low that it did not compromise the studies.

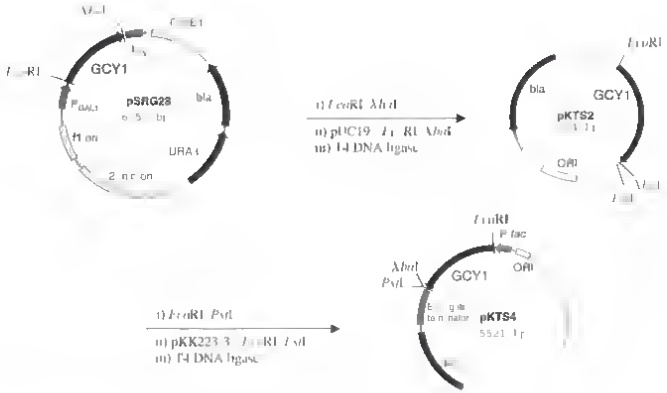
The work on Gcy1p and Gre3p cloning and characterization was realized in cooperation with Kersten T. Schroeder, and the characterization of Ara1p was done in collaboration with Catherine Charron.

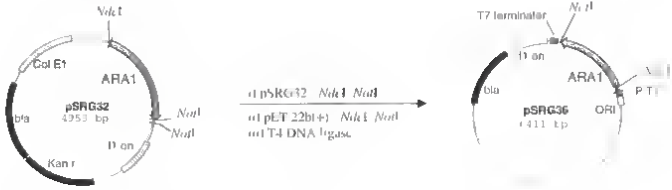
Construction of the *E. coli* Expression Vectors for Gcy1p, Gre3p and Ara1p

The *GRE3* and *GCY1* genes were subcloned into pKK223-3, an expression vector carrying the *tac* promoter (Figures 5-6 and 5-7), while the *ARA1* gene was subcloned into pET-22b(+) where gene expression is driven by the strong T7 promoter (Figures 5-8). Subcloning of *GRE3* and *GCY1* required a two-step strategy: they were first subcloned into pUC19 and finally into pKK223-3. The *GRE3* gene was excised from pSRG26 as an

*Eco*RI *Xho*I fragment and [illegible] into the *Eco*RI and *Sal*[illegible] pKTS1. The gene was then released from this plasmid with *Eco*RI and *H*[illegible] insertion between the same sites in pKK223-3 and providing the *E. coli* expression pKTS3 (Figure 5.6). A similar strategy was used to incorporate a *Pst*I site at the end of the *GCY1* gene that allowed the final cloning into pKK223-3 resulting in the expression vector pKTS4 (Figure 5.7). The *ARA1* gene was isolated from pSRG32 and subcloned into pET 22b(+) as an *Nde*I *Not*I cassette furnishing the expression vector pSRG36 (Figure 5.8).


Col E1
GRE3
bla
pSRG26
6911 bp
f1 ori
URA3
2 micron
i) EcoRI XhoI
ii) pUC19 EcoRI SalI
iii) T4 DNA ligase
pKTS1
3651 bp
ORI
i) EcoRI PstI
ii) pKK223 3 EcoRI PstI
iii) T4 DNA ligase
P tac
Eco term
pKTS3
5568 bp


GCY1
pSRG28
bla
URA3
2 micron
i) EcoRI XhoI
ii) pUC19 EcoRI XhoI
iii) T4 DNA ligase
bla
GCY1
pKTS2
ORI
i) EcoRI PstI
ii) pKK223-3 EcoRI PstI
iii) T4 DNA ligase
EcoRI
P tac
ORI
XhoI
PstI
GCY1
pKTS4



[illegible] Vector construction [illegible] pET 22b(+)

These three [illegible] required different *E. coli* hosts: thus A[illegible] expressed in the *E. coli* BL21 (DE3) strain while Gre3p and Gcy1p were expr[illegible] *coli* JM105. The recombinant strains were used to test the substrate specificity [illegible] expressed enzymes by cell free enzymatic assays and by whole cell biotransf[illegible]

Cell Free Enzyme Activity Assay for Gre3p, Gcy1p and Ara1p

The NADPH cofactor requirement of these enzymes allowed studying the activity towards different substrates by means of a rapid spectrophotometric [illegible] Consumption of this cofactor can be monitored by the decrease in absorbance [illegible] nm [151]. The soluble enzyme fraction from each strain, prepared after induction with isopropyl thio-β-D-galactoside (IPTG), was incubated with the different substrates in presence of NADPH and the course of the reaction was followed by measuring the absorbance at 340 nm.

These studies revealed that Gcy1p, Gre3p and Ara1p reduced α-carbonyl compounds like ethyl pyruvate and 2,3-hexanedione. Although these substrates were also partially reduced also by the non-recombinant control strains, the specific activity was much higher for those strains expressing the yeast reductases (Table 5.3).

Table 5.3 Relative specific activity (SA) of the different crude extracts towards carbonyl compounds (The specific activity has been normalized with respect to the appropriate control strain (data is expressed as a ratio = SA recombinant/ SA control)

Substrate	Over-expressed enzyme		
	Gcy1p	Gre3p	Ara1p
	3.23	3.24	37
	3.72	3.33	28
 **35**			44

Among those transformations the reduction of the β-lactam **35** by the strain over-expressing Ara1p is of particular interest given the synthetic route to the Paclitaxel side chain recently reported by our group (Figure 5-9) [152]. The use of normal baker's yeast as a biocatalyst for this transformation resulted in incomplete stereoselectivity which hampered the utility of this synthetic strategy. Studies with the fas2 deficient mutant ATCC 26403 indicated that FAS was the major enzyme responsible for formation of the *trans* (3*R*,4*R*) isomer[152] however the enzyme(s) responsible for producing the other diastereomers was not known.

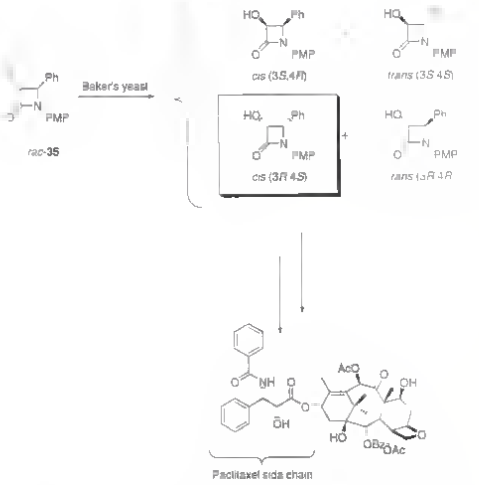
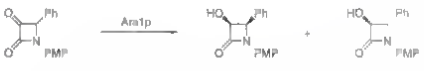


Figure 5.9 Synthesis of the Paclitaxel side chain based on a β-lactam derivative

Since Gre3p, Gcy1p and Ara1p presented activity towards α-carbonyl compounds, the three enzymes were tested for reduction of β-lactam **35** in preparative scale reactions. Crude extracts of *E. coli* strains expressing each enzyme were incubated with 30 mg of substrate in the presence of NADPH and a regeneration system composed of glucose 6-phosphate and glucose 6-phosphate dehydrogenase. The strain expressing

Ara1p was the only one that showed significant activity and the *cis* (3*S* 4*R*) and (3*S* 4*S*) diastereomers were formed in a 57 43 ratio (Figure 5-10). The presence of two diastereomers was detected through chiral HPLC and confirmed by ^{1}C NMR. Unfortunately none of the products was the desired *cis* (3*R* 4*S*) isomer. Interestingly the *trans* isomer produced by Ara1p is not produced when **35** is reduced by whole cells of unmodified baker's yeast. This could mean that other enzyme is the responsible for production of the *cis* (3*S* 4*R*) product in whole cells of unmodified baker's yeast or that Ara1p presents higher commitment to catalysis towards the **4*R*-35** enantiomer. A time course study of the reaction could further clarify the specificity of this enzyme. If Ara1p is the major enzyme producing the *cis* (3*S* 4*R*) diastereomer deletion of the ARA1 gene from the *Δfox2* mutant strain will likely result in a strain with increased stereoselectivity towards formation of the *cis* (3*R* 4*S*) synthon required for the reported synthesis.



rac-**35** *cis* (3*S*,4*R*) *trans* (3*S* 4*S*)

Figure 5-10 Reduction of the β lactam derivative **35** by the *E. coli* strain expressing Ara1p (Free-cell extract and an NADPH regeneration system were used for this reaction).

Whole Cell Biotransformations with *E. coli* Strains Expressing Gcy1p and Gre3p

The substrate specificities of Gcy1p and Gre3p were also analyzed using whole cell biotransformations with the *E. coli* strains expressing these enzymes. In both of these

strains protein expression is under control of the *tac* promoter and inducible by adding 2 IPTG to the culture In contrast to the results obtained with analogous *S. cerevisiae* strains the results with whole cell-mediated reductions indicated that both Gcy1p and Gre3p reduced β-keto esters to β-hydroxy esters with the same absolute configuration as those produced by Ypr1p-mediated reductions

Whole cells of the two strains were tested for the reductions of several β keto esters (Figure 5 11) and the results are summarized in Table 5-4 The optical purities of the products were determined by chiral-phase GC and optical rotations were used to establish the absolute configuration of each β-hydroxy ester product. ^{13}C NMR spectra confirmed that only a single diastereomer was produced in all cases Both strains yielded 3*S*-alcohols with high enantiomeric excess values and acceptable yields for all the substrates tested When racemic α substituted β-keto esters were employed as substrates the facile racemization at the alpha position allowed for dynamic kinetic resolutions (Figure 5 11) [153] Reductions of these substrates with either recombinant strain resulted in almost complete conversion of the substrate to the *syn* (2*R* 3*S*) diastereomer

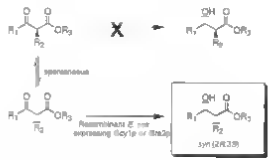


Figure 5 11 Reductions of β keto esters by recombinant *E. coli* strains expressing Gre3p and Gcy1p

Table 5.4 Reductions of β keto esters by recombinant *E. coli* strains expressing Gre3p and Gcy1p

Substrate			*E. coli* expressing Gcy1p		*E. coli* expressing Gre3p		Product characterization	
R_1	R_2	R_3	*Isolated*[4] *Yield*	% ee, de[5]	*Isolated*[4] *Yield*	% ee, de[5]	Absolute Config.	$[\alpha]_D^{25}$ (*c*, $CHCl_3$)
Me	H	Me	28 %	> 98 —[6]	35 %	> 98 —[3]	*S*	+ 28.5 (c 1.5)
Et	H	Me	45 %	91 —	40 %	90 —	*S*	+ 20.5 (c 1.31
Me	H	Et	51 %	> 98 —	45 %	> 98 —	*S*	+ 44.6 (c 1.01
Et	H	Et	48 %	96 —	40 %	91 —	*S*	+ 23.3 (c 1.01
Me	Me	Et	68 %	> 98 > 98	81 %	> 98 > 98	(2*R* 3*S*)	+2.3 (c 5.4)
Me	Et	Et	75 %	> 98 > 98	41 %	> 98 > 98	(2*R* 3*S*)	+ 6.8 (c 7.9)
Me	Allyl	Et	66 %	> 98 > 98	72 %	> 98 > 98	2*R* 3*S*)	+ 3.7 (c 4.6)
Me	Propargyl	Et	56 %	> 98 > 98	87 %	> 98 > 98	2*R* 3*S*)	+7.4 (c 4.11

[4] Yields refer to chromatographically purified samples

[5] Yields of enantiomeric and diastereomeric excess were determined by chiral phase GC a[illegible]

[6] Diastereoselectivity is not applicable [illegible]

These two-new-enzyme cell biocatalysts complement the existing methodologies for reductions of β keto esters by providing simple access to *syn* (2*R* 3*S*) α alkyl β-hydroxy esters in high optical purities. Catalytic hydrogenation of α substituted β keto esters has been limited to formation of the (2*R* 3*R*) and (2*S* 3*R*) diastereomers.[42-44] The (2*S* 3*S*) *anti* diastereomer has been obtained through stereoselective alkylation of ethyl (*S*)-3-hydroxybutanoate.[43] Lewis acid mediated reductions have produced the *syn* diastereomer; however the enantioselectivity of these reagents was not reported.[26] A further advantage of these new biocatalytic reagents is their ability to provide good stereoselectivities even in the absence of bulky substituents which is not always the case for chemical methods of β keto ester reduction. Using whole cells of the engineered *E. coli* strains two stereocenters can be set in one reaction from simple commercially available starting materials. In addition the reaction proceeds under mild conditions and the process is environmentally friendly.

The results obtained from expression of Gcy1p and Gre3p in *E. coli* clearly indicated that these enzyme reduce β-keto esters however the results obtained earlier with the recombinant yeast designed to over-express each of these reductases indicated the opposite. A possible explanation for these results is that even though these enzymes reduce β keto esters their contribution to baker's yeast mediated reduction of these substrates is very low and their over-expression is not noticeable. Thus expression of the enzymes in higher levels and in a host with low native reductase activity such as *E. coli* allowed the characterization of these enzymes. A more likely explanation however is that expression of these enzymes is tightly regulated in yeast and the cell either degrades the expressed enzyme or counteracts its activity. Evidence of yeast control on expression

of β-keto esters [redacted]

particular enzyme activity (Chapter 3)

Summary

The results presented here indicate that the identi[redacted] by detecting yeast proteins with sequence similarity to known [redacted] and powerful approach. The studies of three aldo keto reductases with high [redacted] with Ypr1p resulted in the characterization of three carbonyl reductases able [redacted] carbonyl compounds. Of particular importance is the ability of Ara1p to reduce β-[redacted] 35, since this information can be used in the design of novel yeast strains [redacted] of the Paclitaxel side chain. Among the studied enzymes Gcy1p and Gre[redacted] reduce β-keto esters to yield β-hydroxy esters with the same absolute configurat[redacted] those produced by Ypr1p mediated reduction.

The construction of recombinant *E. coli* strains expressing Gcy1p in [redacted] provided simple and inexpensive biocatalysts that can be used by non specialist. [redacted] approach eliminates the need for enzyme isolation and cofactor regeneration, and the [redacted] new biocatalysts broaden the spectrum of reactions that can be catalyzed with recombinant *E. coli* cells.

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

This project has provided a better understanding of the yeast enzymes involved in β-keto ester reductions. Three enzymes had been previously reported as the participants in the reduction of β-keto esters by baker's yeast [69 94 105 106]. The results obtained from this work proved that although those enzymes were involved to an important extent in these reductions, they were not the only players. Two other β-keto ester reductases, Gcy1p and Gre3p, were identified during the course of this work, and the results from the mutant strains indicate that even more enzymes are involved in these reactions. In addition, the isolation of a major β-keto ester reductase resulted in the identification of Gre2p as the α-acetoxy ketone reductase. This finding corrected a previous mis-identification[109 115] and allowed the construction of strains required for this work.

The rational design of yeast strain also provided useful biocatalysts. Yeast strains with high stereoselectivity were obtained by combining knock-out and over-expression of the three previously reported enzymes. Strain 2B(pSRG41) provided access to the optically pure *S* alcohols, and strains JS3(pSRG41) and 10495B(pSRG14) provided complete stereoselectivity in the synthesis of some α-substituted β-hydroxy esters. For one of the substrates, both the *syn*- and *anti*- diastereomers could be prepared in pure optical form depending on the engineered yeast strain employed. These results illustrate

[redacted] approach to tailor the stereospecificity of baker's [redacted] the engineered strains provided comparable or better results than those obtained with previously reported methodologies [97 98 100 120 122] and it constitutes a general strategy that uses a low mass of cell and does not require the use of inhibitors

In addition to the engineered yeast strains, two recombinant *E. coli* strains expressing Gcy1p and Gre3p have been constructed and provide simple access to (*2R 3S*) α alkyl β hydroxy esters in high optical purities. Using whole cells of the engineered *E. coli* strains, two stereocenters can be set in one reaction from simple, commercially available starting materials.

The new biocatalysts prepared during this project complement the existing methodologies for reduction of β keto esters by providing simple access to β hydroxy esters in high optical purities. They provide simple and inexpensive biological reagents that can be used by non specialists. This approach eliminates the need for enzyme isolation and cofactor regeneration, and broaden the spectrum of reactions that can be catalyzed with recombinant *E. coli* or yeast cells.[73 74 155] A further advantage of these new biocatalytic reagents is their ability to provide good stereoselectivities in the absence of bulky substituents, which is not always the case for chemical methods of β-keto ester reduction. In addition, the reactions proceed under mild conditions, and the processes are environmentally friendly.

Further rational design of yeast strains to tailor the stereoselectivity of yeast mediated reductions requires the identification of all the relevant enzymes involved in those reactions. Homology studies have indicated that there are close to 50 enzymes with sequence similarity to known reductases.[104] The identification of Gcy1p, Gre3p, and

Although based on the [illegible] with known reductases and results [illegible] expression in *E. coli* argues that this could be a useful and powerful approach [illegible] identification of other reductases. Particularly interesting is the study of the Gre3p closely related to Gre2p (Table 5.1) since they are likely to present the opp[illegible] stereoselectivity than the aldo-keto reductase family and may provide a useful [illegible] the synthetic approach to the Paclitaxel side chain (Figure 5.7). Cloning and ex[illegible] of these reductases is an ongoing project in our laboratory.

The construction of recombinant *E. coli* strains expressing each of the [illegible] yeast reductases could provide easier access to useful biocatalysts than the [illegible] yeast strategy since the latter requires more complicated genetic constructions. However *E. coli* may also present some problems. Although this organism proved a useful host for the expression of some reductases, the yields of the reactions were lower than those obtained with the engineered yeast strains. This may be associated with cofactor availability, a problem that has been encountered previously by other workers. A second potential problem that should be considered relates to post-translational modifications that some enzymes may require. Finally, while the presence of the endogenous reductase activity in *E. coli* did not affect the biotransformations reported in this work, may constitute a problem for reduction of other substrates. An alternative could be the use of a different host such as *Pichia pastoris* for which no reductase activity has been reported; however a preliminary screening for native reductase activity should be done before embarking in the use of any new host.

Finally, exploring the "designer yeast" and *E. coli* strains for reduction of other substrates could uncover novel synthetic applications for the strains and will provide a

[illegible] in baker s yeast Some interesting s[illegible]

which reduction with baker s yeast provides incomplete stereoselectivity inc[illegible]

esters diketones, hydroxy ketones chloro ketones and unsaturated carbonyl c[illegible]

In addition it will be interesting to explore synthetic applications of the obt[illegible]

optically pure β hydroxy esters to the synthesis of drugs or natural products [illegible]

lescribed in the introduction (Figure 1 2)

CHAPTER7
EXPERIMENTAL

Molecular Biology and Yeast Genetics

Biochemicals and Media

Bacto Tryptone, Bacto-Yeast Extract, Bacto Peptone and Bacto Yeast Nitrogen Base without amino acids were purchased from Difco. Dextrose and galactose were obtained from Fisher. Geneticin and chloramphenicol were purchased from Sigma. Ampicillin was obtained from Promega. Amino acids and uracil used as supplements in the media were purchased from Sigma.

Restriction endonucleases were purchased from New England Biolabs or Promega. T4 DNA ligase was obtained from New England Biolabs. Oligonucleotides were obtained from Integrated DNA technology or Genset Biotechnology. *Taq* polymerase, PCR and sequencing grade, as well as the DNA Silver Sequencing kit were purchased from Promega. Agarose was obtained from Kodak. The DIG DNA labeling and detection kit (Genius kit) was purchased from Boehringer-Mannheim.

Vectors

E. coli cloning vectors pUC18, pUC19[156] and pBC SK(+) (Stratagene) were from our laboratory collection. *E. coli* expression vectors pKK223-3 and pET 22b(+) were obtained from Pharmacia and Novagen, respectively.

Yeast cloning vectors YCplac111 and YCplac22 were a generous gift of Dr R D Gietz Plasmid YEp13 was kindly provided Dr H Baker Yeast expression vector pYES2 was purchased from Invitrogen and pAAH5 was generously provided by Dr B Hall [179]

E. coli and *S. cerevisiae* Strain Genotypes

E. coli strains used in this work

E. coli strains XL1 blue and JM109 were used for routine cloning procedures while JM105 and BL21(DE3) were used for protein expression

Table 7.1 *E. coli* strains used in this work and their genotypes

E. coli strain	Genotype
XL1-blue	F' Tn*10* *proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/ *recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*
JM109	F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/ e14 (McrA$^-$) Δ(*lac proAB*) *thi gyrA96* (Nal^r) *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 relA1*
JM105	F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/ *thi rpsL* (Str^r) *endA sbcB15 sbcC*$^?$ *hsdR4* (r_K^- m_K^+) Δ*lac proAB*)
BL21(DE3)	F' *ompT* (*lon*) *hsdS*$_B$ (r_B^- m_B^- an *E. coli* B strain) with DE3 = λ prophage carrying the T7 RNA polymerase gene

S. cerevisiae strains used in this work

The yeast strains used for protein expression were InvSc1 purchased from Invitrogen and 15C generously provided by Dr A Buchman The latter carries a *pep4-3* mutation that results in reduced expression of three proteases RNase and phosphatase The strain ATCC 26403 carrying an uncharacterized mutation within the *FAS2* gene was

purchased from the American Type Culture Collection. A heterozygous diploid carrying the deletion of the same gene was purchased from Research Genetics (strain 21061). Strain 10495B carrying a deletion of the *GRE2* gene was procured from Eurosacrf.

Table 7.2. *S. cerevisiae* strains used in this work and their genotypes

S. cerevisiae strains	genotype
15C	*MATα, leu2, ura3-52, Δtrp1, his4-80, pep4-3*
InvSc I	*MATa/MATα, leu2, ura3-52, Δtrp1, his3*
ATCC 26403	haploid a (-) *fas2*
Research Genetics strain 21061	*MATa/MATα, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0 fas2::kanMX4* (heterozygous to the last three mutations)
10495B or FAI0012 (AL)	*MATa, ura3-52, HIS3, leu2Δ1, LYS2, trp1Δ63, gre2::kanMX4*

S. cerevisiae constructed in this work

The strains JS2 and JS3 carrying a deletion of the *YJR105w* and of the *YPR1* genes, respectively were derived from 15C. The haploid strain 2B deficient in fatty acid synthetase was created by sporulation of the Research Genetics 21061 strain. Strain 24B was created by crossing and sporulation of JS3 and 10495B and carries deletions of both the *YPR1* and the *GRE2* genes.

Table 7.3. *S. cerevisiae* strains created along this work and their genotype.

S. cerevisiae strains	genotype
JS2	*MATa, leu2, ura3-52, Δtrp1, his4-80, pep4-3, ypr105w::LEU2*
JS3	*MATa, leu2, ura3-52, Δtrp1, his4-80, pep4-3, ypr1::URA3*
2B	*MATa, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0, fas2::kanMX4*
24B	*MATa, HIS3, his4, leu2, LYS2, trp1, gre2::kanMX4, ypr1::URA3*

Maintenance of *E. coli* and *S. cerevisiae* Strains.

Standard media (LB) and techniques for routine growth and maintenance of *E. coli* strains were used [157]. Recipe per liter: Bacto-Tryptone (10 g), Yeast Extract (5 g), NaCl (10 g). For solid media, agar (15 g) was included. When appropriate, ampicillin was added to 200 ug/mL or chloramphenicol was used at concentrations of 170 ug/mL.

Non-selective yeast growth medium (YPD) was used for routine growth and maintenance of *S. cerevisiae* strains. Recipe per liter: Bacto-Yeast Extract (10 g), Bacto-Peptone (20 g), and dextrose (20 g) [158]. Agar (20 g/L) was included for preparation of solid media. For geneticin-resistant strains, geneticin (G418) was added at a concentration of 200 ug/mL. Selective media was used for routine maintenance of recombinant strains. Recipe per liter: Bacto yeast nitrogen base without amino acids (6.7 g), dextrose (20 g), and the apropriate amino acids or uracil according to the auxotrophic marker present in the strain: L-tryptophan (20 mg/L), L-histidine (20 mg/L), L-leucine (30 mg/L), L-methionine (20 mg/L), L-lysine (30 mg/L), and uracil (2 g/L). For solid media, agar was added (20 g/L).

For short-term storage, yeast and bacterial strains were kept on plates sealed with parafilm at 4 °C. For long-term storage strains were stored at –80 °C in media containing 15% glycerol.

Recombinant DNA Techniques

Recombinant DNA procedures were carried out essentially as described by Sambrook et al.[157] *S. cerevisiae* DNA used as template in the PCR reactions and as control in the southern blots was isolated following the medium scale protocol reported by Wach et al.[158] Genes were PCR amplified using a Perkin Elmer Geneamp PCR system 2400, purified by agarose gel electrophoresis and digested with appropriate restriction enzymes for cloning into the desired vector. Restriction enzyme digestions were checked by DNA gel electrophoresis. DNA agarose gel electrophoresis was carried out as described by Sambrook.[157] *E. coli* transformations were performed by electroporation while yeast transformations utilized the high efficiency lithium acetate protocol.[147] Purified DNA for sequencing and yeast transformation was obtained by density gradient ultracentrifugation with CsCl in presence of ethidium bromide (plasmids pSRG1-pSRG22) or by means of the Clontech or QIAGEN midiprep kits (plasmids pSRG23-pSRG41). DNA sequencing was accomplished using the Silver Sequence kit according to the manufacturer's instructions. Southern blots were done with DIG DNA labeling and detection kit, procedure and probe labeling followed manufacturer's instructions.

Yeast Genetic Manipulations

Media

The presporulation and sporulation media described by Kaiser et al were used for Presporulation medium, recipe per liter: Bacto-Yeast Extract (8 g) Bacto-Peptone (3 g) dextrose (100 g) and Bacto-agar (20 g) Sporulation medium recipe per liter: potassium acetate (10 g) Bacto-Yeast Extract (1 g) dextrose (0.5 g) and Bacto-agar (20 g) For sporulation of auxotrophic diploids, nutritional supplements were added at 25 % level of those used for selective media

Procedures

Mating experiments

Diploid strains were generated by mating of the parental strains The two parental strains were plated in single lines that crossed at the middle of the plate in media where only the diploid strain could grow After 4-5 days, colonies of the diploid strain developed at the intersection of the two cultures

Sporulation and ascI dissection

The diploid strains were grown for two days in solid presporulation media and then transferred to solid sporulation media where they were grown from 4-15 days to allow spore formation A small dab of the culture was taken periodically and examined for spore formation under a microscope Once spores were formed, a small quantity of the sporulated culture was resuspended in 100 uL of zymolase solution (0 5 mg/mL in 1 M sorbitol) and incubated for 15 – 20 minutes at room temperature Then 0 5 mL of sterile water was carefully added, trying to avoid breaking the spores A drop of the digested asci

solution was transferred to a YPD plate (with fatty acids if necessary) and spread in a single line across the middle of the plate. Asci dissections were performed under a microscope equipped with a micromanipulator. A cluster of 4 spores was selected, the four spores were separated and moved to isolated positions in the YPD plate as described by Kaiser et al.[160] The spores usually germinated and formed colonies within a period of 2-3 days after dissection. The phenotypes were determined by replica plating onto appropriate media. The mating types were determined by crossing with appropriate MATa and MATα strains.

Isolation of Gre2p

Materials and Equipment

Baker's yeast was obtained from Publix Bakery and was the product of Gist Brocades (Delft, Holland). DEAE sepbacel was obtained from Sigma. Hypatite C from Clarkson Chemical Company, and Sephacryl S-200 from Pharmacia. Matrex Blue-A, Red-A and the PM-10 ultrafiltration membranes were purchased from Amicon. Benzamidine, p-tolnensulfonylfluoride (PMSF) and ethyl 4-chloro acetoacetate were procured from Aldrich. Dithiothreitol (DTT) was purchased from Promega. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH and NADP were obtained from Sigma.

An SLM-AMINCO French pressure cell was used for cell disruption. Ultracentrifugations were performed in a Beckman L5-65 ultracentrifuge. Column chromatography steps utilized a Dynamax RP-1 peristaltic pump (Rainin) and the elution

profile was followed by an ISCO UA-5 recorder equipped with a UV detector [illegible] assays were performed in an Olis Cary 14 UV vis spectrophotometer

Experimental Procedures

All the enzyme purification procedures were carried out at temperatures between 0 and 4 °C. All the buffers used during the isolation contained 5% glycerol, 0.34 g/l benzamidine, 0.3 g/l PMSF and 1 mM DTT unless otherwise stated. PMSF and DTT were added to the buffer immediately before use. The buffer used for cell lysis also contained 1mM EDTA.

Preparation of the Crude Extract

The dry Bakers' Yeast (250 g) was hydrated in deionized water and 2x0 [illegible] of the final wet mass (750 g) were used. The cells were washed with three volumes of deionized water and collected by centrifugation at 1-000 g for 10 min. This process was repeated twice and the washed cells were suspended in ice cold 0.2 M potassium phosphate buffer (pH 7.4). The cells were disrupted by a French pressure cell at 10 000 to 12 000 psi. The residual yeast cells and membrane pellet were removed by centrifugation at 1 000 × g for 10 min and were resuspended in the same buffer and the process repeated again. The combined supernatants were centrifuged at 100 000 × g for 30 minutes to remove the mitochondrial fraction.

Ammonium Sulfate Precipitation

To the clear cell extract, finely ground ammonium sulfate powder was added until the desired concentration was achieved. The mixture was then stirred for 20 minutes and the precipitated fraction separated by centrifugation at 10 000 × g for 20 minutes. The

[redacted] was repeated with the supernatant until the next level of saturation was achieved. The procedure was repeated twice to obtain the 50-80 % $(NH_4)_2SO_4$ saturation fraction reported to have all the reductase activity.[37] Activity assays demonstrated that this was indeed the case and all the reductase activity was precipitated among within this range of saturation. The protein fraction was dissolved in 10 mM Tris-HCl buffer (pH 7.5) and dialyzed against 4L of the same buffer with one change of buffer overnight.

Concentration of Enzyme Fractions

The protein samples obtained from each purification step were concentrated by means of an Amicon ultrafiltration kit fitted with a PM-10 membrane under nitrogen at 40 – 60 psi.

Protein Determinations

Protein concentration

A chart recorder of A_{280} displayed the elution pattern from each column chromatography step. A Bradford assay[161] of the concentrated samples allowed determination of the total protein present in the fractions with reductase activity.

SDS gel electrophoresis

SDS gel electrophoresis was used to monitor the purification steps and it was carried out essentially as described by Maniatis.[157] Gels were stained with Coomassie Brilliant Blue R for 30 minutes at room temperature and destained overnight. A special protocol was required for proteins used for amino acid sequencing (*vide infra*).

Enzyme Assays

Enzyme activity was determined spectrophotometrically at 30 °C by following the decrease in absorbance of NADPH at 340 nm. A 25 µL aliquot of chromatographic fraction was added to 1.5 mL of a solution of 0.10 M phosphate buffer (pH 7.0), 0.2 M KCl that also contained ethyl 4-chloro acetoacetate (0.98 mM) and NADPH (0.09 mM). The high salt concentration in the buffer was essential to avoid protein precipitation. One unit of enzyme was defined as the amount of enzyme consuming 1 µmol of NADPH per minute under the assay conditions. Total units were calculated from the initial rate of NADPH consumption. The slope from the spectrophotometric assays is ΔA_{340}/min and can be correlated to µmoles of NADPH (ε=6200)

$$\text{UNITS} = 240 \times \text{slope} \times \text{fraction V (mL)/assay V}_{\text{total}} \text{ (mL)}$$

Biocatalysis with the Enzyme Fractions

A typical reaction mixture contained 3 mg of NADP, 85 mg of glucose 6-phosphate, 0.1 mg of glucose 6-phosphate dehydrogenase, 30 mg of substrate, and a suitable quantity of enzyme preparation in a total volume of 20 mL enzyme assay buffer. The substrate was dissolved in a small amount of ethanol prior addition to the reaction. The reaction was stirred at 30 °C for 48 h. The extent of conversion and the values of enantio- or diastereomeric excess were determined by gas chromatography as described under biotransformations.

Sequencing

The sequencing and amino acid analysis of the Gre2p was performed by the University of Florida ICBR Protein Core and sample preparation followed their

recommended protocols [8]. SDS PAGE electrophoresis were carried out as described earlier with slight differences. Samples for amino acid analysis and sequencing were prepared in Laemmli buffer. The sample for amino acid analysis was blotted onto a PVDF membrane using 10 mM MES pH 6.20% MEOH as buffer and transfer was carried on overnight at 20 volts in a cold box. The membrane was stained in 0.02% Coomassie blue 40% methanol 5% acetic acid for 20-30 sec and destained for one minute in 40% methanol 5% acetic acid. After staining the membrane thoroughly in distilled water it was air dried, placed between Whatman filter papers wrapped in foil and sent for amino acid analysis. The gel for internal sequencing was loaded with 300 – 400 pmoles of protein. Staining of the gel was performed in the same manner as for the PVDF membrane and destaining was conducted overnight. The gel slice containing the protein was sent for internal sequencing.

The sample for amino acid analysis was hydrolyzed in 6N HCl and analyzed by the pre-column derivatization method using PITC on the Applied Biosystem 420A. For internal sequencing the protein sample was cleaved with endoproteinase Lys-C. The generated peptides were separated by small bore HPLC (reverse phase on a Vydac C18 column) and subject to N-terminal sequence analysis by Edman degradation chemistry in a Procise (Applied Biosystems models 494 HT) sequencer.

Organic Synthesis

Chemicals and General Procedures

Reagents were obtained from commercial suppliers and were used as received. Unless otherwise specified, reactions were carried on flame dried glassware under an

solvents and under a stream of argon. In general, reaction products were purified by flash chromatography using 60A silica gel. Thin layer chromatography was performed on [illegible] of silica gel 60 from Aldrich.

Product characterization was done by GC/MS, proton and carbon NMR, IR, and optical rotations if applicable. NMR spectra were taken on a Varian Gemini or VXR300 instruments operating at 300 MHz. All spectra were obtained in $CDCl_3$ and referenced to residual $CHCl_3$ (7.26 ppm) for ^1H NMR and to $CDCl_3$ (77.0) for ^{13}C NMR. IR spectra were recorded from thin films on a Perkin-Elmer 1600 FT IR spectrophotometer. GC/MS was taken on a Hewlett Packard 5890 series II equipped with a mass selective detector. Capillary gas chromatography was performed in a Hewlett Packard 5890A equipped with a flame ionization detector. A 0.32mm x 30m DB-17 column was used for non chiral separations, while a Chrompack 0.25mm x 25m CP chirasil-Dex CB column was used for chiral separations. The gradient used for the DB-17 column was: 80 °C (5 min) to 180 °C (5 min) at 10 °C/min. For the chiral separations two different sets of conditions were used depending on the substrate (*vide infra*). The injector and detector temperatures were maintained at 250 °C and 220 °C respectively. Optical rotations were measured from $CHCl_3$ solutions using a Perkin Elmer 341 polarimeter operating at room temperature.

Synthesis of Substrates

With the exception of ethyl α-allyl acetoacetate and ethyl α-propargyl acetoacetate, the substrates were obtained from Aldrich. Synthesis of these two substrates was performed as reported by Dauben et al[163] with slight modifications. The reaction was performed in a three neck round bottom flask equipped with an addition funnel and an elbow tube for solid addition. First, ethyl acetoacetate (38.4 mmoles) was dissolved in 20

mL DMF and the reaction was placed in an ice bath. Solid NaH (42.3 mmole) was slowly added to the stirred ethyl acetoacetate solution. This reverse addition favored the formation of the mono-anion over the di-anion, avoiding di-alkylation. After addition of the NaH was completed, a solution of the corresponding alkyl bromide (42 mmole) in 10 mL DMF was added dropwise over a period of 30 minutes. The reaction was kept at 0 °C for another half an hour and then brought to room temperature and stirred over a period of 24-48 hours. The reaction was followed by TLC and allowed to reach completion. After the reaction was completed, the flask was placed in an ice bath and acetic acid was added to quench excess of NaH. The mixture was dissolved in brine and extracted with diethyl ether. The ether layer was then washed with brine, dried with Na_2SO_4 and the solvent was removed by rotary evaporation. The crude material was purified by either flash chromatography with CH_2Cl_2:hexane 7:3 or by vacuum distillation. This procedure resulted in a crude yield of 60-65 % and isolated yields of 35 % to 44 %.

Synthesis of Racemic β-Hydroxy Ester Standards

Racemic β-hydroxy esters used as standards in GC analysis were obtained by $NaBH_4$ reduction of the corresponding β-keto ester.[164] The reactions were carried out in ethanol for the ethyl esters and methanol for the methyl esters to avoid trans-esterification. Sodium borohydride (1.1 equivalents) was dissolved in the appropriate alcohol to provide a 10-15 % solution. The β-keto ester was then added dropwise to the stirred solution of $NaBH_4$ at room temperature. The progress of the reaction was followed by TLC and allowed to achieve completion (overnight). Excess of $NaBH_4$ was destroyed

by adding acetic acid and the alcohol was [illegible] ml). The [illegible] solution was then diluted in a $NaHCO_3$ sol[illegible] and ethyl ether [illegible] times). The ether layer was then washed with br[illegible] with $NaHCO_3$ [illegible] was removed by rotary evaporation. The crude material was purified by flash chromatography with the solvent system ether-hexane 1:1. The isolated yield [illegible] between 60 and 70 %.

Acetylation of β-Hydroxy Esters

For most β-hydroxy esters, the different alcohol stereoisomers were resolved by chiral phase GC with no need for derivatization. However, methyl 3-hydroxyhexanoate and ethyl 2-ethyl-3-hydroxybutanoate required prior acetylation for good resolution of the optical isomers. Quantitative acetylation was achieved with acetic anhydride in DMF using DMAP (dimethyl amino pyridine) as the base.[165] In a normal procedure, 5 mg of the β-hydroxy ester was dissolved in 300 µL DMF, then 3 µL acetic anhydride and a small crystal of DMAP were added to the solution and the reaction was stirred overnight. After completion of the reaction, one mL of 0.5 M $NaHCO_3$ solution was added to the reaction mixture followed by extraction with 2 mL of ether. The extract was diluted two times and used for GC analysis.

Biotransformations

General Procedure for Biotransformations with Yeast Strains

The different strains of *S. cerevisiae* were maintained on SD plates with the appropriate nutrients added (*e.g.* 2B and 2B(pSRG41) required fatty acid supplementation). Fresh plates were streaked from a frozen stock and a single colony was

Inoculate 50 mL of YPD in a sterile 250 mL Erlenmeyer flask. The culture was incubated at 30 °C in a rotary shaker at 200 rpm until the OD_{600} value was between 4 and 6. Cells were then harvested by centrifuging at 3000 × *g* for 10 min. The cell pellet was resuspended in 20 mL of 10 mM Tris-Cl, 1 mM EDTA (pH 7.5) by vortexing. This washing procedure was repeated an additional two times. The final cell pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA (pH 7.5), 15% glycerol at a concentration of 0.1 g/mL (wet weight). At this stage, cells were either used directly for a reaction or frozen in aliquots at –80 °C for later use. Standard reaction mixtures for preparative biotransformations contained 90 mL YP, 10 mL 20% galactose or glucose, depending on the inducer, and 10 mM substrate. Freshly prepared or frozen cells were added to a final concentration of 2 mg/mL. Reaction flasks were shaken at 200 rpm and 30 °C, and the conversion was monitored by GC. Analytical samples were prepared by mixing 200 µL of the reaction mixture with 600 µL of ethyl acetate. After vortexing for 10 sec, the mixture was centrifuged in a microcentrifuge for 2 min, the organic layer was removed, and 1 µL was used for GC analysis. After the reaction was complete, the reaction mixture was centrifuged at 3000 × *g* for 10 min at 4 °C to remove yeast cells. The supernatant was extracted with CH_2Cl_2 (3 × 50 mL). The cell pellet was extracted with 20 mL of CH_2Cl_2, and the organic extracts were combined, washed with brine, and dried with Na_2SO_4. When emulsions formed, they were broken by centrifugation at 3000 × *g* for 10 min. After concentrating the sample by rotary evaporation, the β-hydroxy esters were purified by flash chromatography on a 1 × 15 cm silica column using 1:1 ether:hexanes as mobile phase.

General Procedure for Biotransformations with Recombinant *E. coli* Strains

Fresh plates of engineered *E. coli* strains were streaked from the appropriate frozen stock and a single colony was used to inoculate 10 mL of LB-ampicillin in a 125 mL Erlenmeyer flask. The culture was incubated in a rotary shaker overnight (200 rpm, 37 °C) and 1 mL of this culture was used to inoculate 100 mL fresh LB ampicillin in a 500 mL Erlenmeyer flask. The fresh culture was grown under the same conditions until it reached an OD_{600} of 0.5 (approximately 2.5 hours), then IPTG was added to a final concentration of 1mM along with the substrate (10 mM). The culture was shaken (200 rpm) at room temperature and sampled periodically for GC analysis. After maximum conversion was achieved (24 – 50 hours), the cells were removed by centrifugation at 4 000 × g for 15 minutes at 4 °C. The supernatant was extracted with CH_2Cl_2 (4 × 50 mL), the combined organic extracts were washed with brine, dried with Na_2SO_4, filtered, and evaporated. The product was purified by flash chromatography with $CHCl_3$–ethyl ether (9:1) as the solvent system. The products were characterized by IR, NMR, GC–MS, and optical rotations.

Chiral-Phase GC Analysis of the β-Hydroxy Esters

Enantiomeric and diastereomeric excess values were determined by chiral phase GC. Chiral separations used the following conditions: 70 °C (2 min) to 130 °C (5 min) at 1.0 °C/min, followed by a 10 °C/min to 180 °C (10 min), except for ethyl 3-hydroxybutanoate and ethyl 2-methyl-3-hydroxybutanoate for which the ramp was started at 60 °C and finished at 110 °C. To determine the retention times for all the optical isomers and to standardize conditions for good resolution, racemic mixtures were used. Methyl benzoate was used as an internal standard. For most products, the different

ethyl stereoisomers were resolved under these conditions, however methyl 3-hydroxybutanoate and ethyl 2-ethyl 3-hydroxybutanoate required prior derivatization for good resolution.

NMR Data for Racemic and Optically Enriched β Hydroxy Esters

After purification by flash chromatography, reaction products were characterized by optical rotation, IR, GC-MS, ^{1}H NMR, and ^{13}C NMR when appropriate. All the hydroxyesters are known compounds, and the spectral data agreed with literature reports.[43,101,166] In the case of ethyl 2-alkyl 3-hydroxy esters the spectral data provided information on the diastereomeric purity.

Methyl 3-hydroxybutanoate

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.21 (1H, m), 3.72 (3H, s), 3.12 (1H, OH, s), 2.49-2.56 (2H, AB q, J_{gem}= 16.2 Hz, J_{vic} = 4.6 and 8.1 Hz), 1.23 (3H, d, J = 6.32 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 174.0, 69.56, 51.88, 40.94, 29.61, 9.96.

Methyl 3-hydroxypentanoate

^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.91(1H, m), 3.68 (1H, s), 2.32-2.53 (2H, AB q, J_{gem} = 16.3 Hz, J_{vic} = 3.6 and 8.7 Hz), 1.40-1.60 (2H, m), 0.94 (3H, t, J = 7.48 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 173.8, 69.57, 52.0, 40.9, 29.6, 10.1.

Ethyl 3-hydroxybutanoate

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.16 (2H, q, J = 7.2 Hz), 3.92(1H, m), 3.14 (1H, OH, s), 2.35-2.55 (2H, AB q, J_{gem}= 16.4 Hz, J_{vic} = 4.2 and 8.4 Hz), 1.26 (3H, t, J = 7.2 Hz), 1.21 (3H, d, J = 6.3 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 174.0, 60.6, 42.67, 23.16, 14.1, 13.2.

Ethyl 3-hydroxypentanoate

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.16 (2H, q, J = 7.33 Hz), 3.92 (1H, m), 3.08 (1H, OH, s), 2.33-2.54 (2H, ABq, J_{gem} = 16.36 Hz, J_{vic} = 3.17 and 9.04 Hz), 1.51 (2H, m), 1.26 (3H, t, J = 7.08 Hz), 0.95 (3H, t, J = 7.33 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 173.43, 69.59, 60.93, 41.09, 29.61, 14.42, 10.08.

Ethyl 3-hydroxyhexanoate

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.15 (2H, q, J = 7.08 Hz), 4.0 (1H, m), 3.12 (1H, OH, s), 2.33-2.53 (2H, ABq, J_{gem} = 16.6 Hz, J_{vic} = 3.42 and 8.79 Hz), 1.34-1.54 (4H, m), 1.25 (3H, t, J = 7.08 Hz), 0.92 (3H, t, J = 6.83 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 173.43, 67.99, 60.94, 41.54, 38.86, 18.93, 14.42, 14.22.

Ethyl 2-methyl-3-hydroxybutanoate (racemic)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.15 and 4.16 (2H, double q, J=7.02Hz), 4.04 and 3.86 (1H, m), 2.65 (1H, s), 2.37-2.53 (1H, m), 1.24 (3H, t, J = 7.08 Hz), 1.16 (3H, d, J = 7.08 Hz), 1.14 (3H, d, J = 6.59 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 175.8, 69.6, 67.91, 65.55, 60.8, 47.2, 45.43, 20.9, 19.70, 15.21, 14.4, 14.06, 11.3.

Ethyl 2-ethyl-3-hydroxybutanoate (racemic)

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.17 and 4.16 (2H, double q, J=7.18 Hz), 3.83-4.01 (1H, m), 2.55 (1H, OH), 2.22-2.36 (1H, m), 1.60-1.77 (2H, m), 1.26 (3H, t, J=7.18 Hz), 1.16 and 1.19 (3H, double d, J=6.32 Hz), 0.92 and 0.91 (3H, double t, J=7.42 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 175.51, 175.19, 68.34, 60.69, 54.56, 54.33, 22.91, 21.82, 20.95, 20.73, 14.64, 12.47, 12.01.

Ethyl 2-allyl-3-hydroxybutanoate (racemic)

H NMR (CDCl3, 300 MHz) δ 5.[illegible] 5.07 (2H, m), 4.16 (2H, q, J [illegible]
4.01 (1H, m), 2.30-2.57 (3H, m), 1.26 (3H, t, J = 7.18 Hz), 1.20 (3H, d, J = 6.4 [illegible]
NMR (CDCl3, 300 MHz) δ 174.7, 135.7, 134.9, 117.4, 117.0, 68.1, 63.9, 60.[illegible]
52.2, 33.9, 32.2, 21.65, 20.6, 14.5

Ethyl 2-propargyl-3-hydroxybutanoate (racemic)

^{1}H NMR (CDCl3, 300 MHz) δ 5.15 (1H, s,b), 4.2(2H, q, J=7.04 Hz), 4.09(1H, m), [illegible]
2.68 (3H, m), 1.96-2.1 (1H, m), 1.27 (3H, t, J=7.04 Hz), 1.21 (3H, d, J=6.45 Hz), [illegible]
NMR (CDCl3, 300 MHz) δ 176.58, 173.76, 81.58, 80.91, 70.58, 70.31, 67.77, 67.[illegible]
51.4, 51.1, 21.23, 20.63, 18.79, 17.33, 14.46

Ethyl 2(*R*) methyl 3(*S*)-hydroxybutanoate

^{1}H NMR (CDCl3, 300 MHz) δ 4.17 (2H, q, J=7.18 Hz), 4.07 (1H, m), 2.6 (1H, [illegible]
1H, d,q, J = 7.28 Hz, J$_{2,3}$ = 3.8 Hz), 1.27 (3H, t, J = 7.18 Hz), 1.19 (3H, d, J = [illegible] Hz
1.18 (3H, d, J = 6.45 Hz). ^{13}C NMR (CDCl3, 300 MHz) δ 176.29, 68.2, 60.9, 45.[illegible]
14.43, 11.3

Ethyl 2(*R*)-ethyl 3(*S*) hydroxybutanoate

H NMR (CDCl3, 300 MHz) δ 4.17(2H, q, J=7.18 Hz), 3.95(1H, m), 2.51 (1H, OH, [illegible]
1H, m), 1.61-1.77 (2H, m), 1.26 (3H, t, J=7.18 Hz), 1.18 (3H, d, J=6.31 Hz), 0.92 (3H, t,
J=7.40 Hz). ^{13}C NMR (CDCl3, 300 MHz) δ 175.5, 68.38, 60.64, 54.36, 20.88, 20.65,
14.53, 12.34

Ethyl 2(*R*) allyl 3(*S*) hydroxybutanoate

H NMR (CDCl3, 300 MHz) δ 5.7-5.9 (1H, m), 5.07 (2H, dd, J$_{cis}$ = 10.2 Hz, J$_{trans}$ = 15.7
Hz, J$_{vic}$ = 1.4 Hz), 4.16 (2H, q, J = 7.18 Hz), 4.01 (1H, m), 2.30-2.57 (3H, m), 1.26 (3H, t,

J = 7.18 Hz) 1.20 (3H, d, J = 6.45 Hz) ^{13}C NMR (CDCl$_3$, 300 MHz) δ 174.7 136.0 117.0 68.1 60.8 52.2 32.1 20.6 14.5

Ethyl 2(*R*)-propargyl-3(*S*)-hydroxybutanoate

^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.21 (2H, q, J=7.14 Hz) 4.11 (1H, m) 2.50-2.70 (3H, m) 2.38 (1H, OH, d, J=4.2 Hz) 2.03 (1H, dd, J=2.44, J=2.69 Hz) 1.3 (3H, t, J=7.14 Hz) 1.24 (3H, d, J=6.45 Hz) ^{13}C NMR (CDCl$_3$, 300 MHz) δ 173.5, 81.6, 70.3, 67.7, 61.24, 51.36, 20.7, 17.3, 14.4

Ethyl 2(*S*)-ethyl 3(*S*) hydroxybutanoate

^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.18 (2H, q, J=7.13 Hz) 3.92 (1H, m) 2.62 (1H, OH) 2.29 (1H, m) 1.60-1.78 (2H, m) 1.27 (3H, t, J=7.13 Hz) 1.21 (3H, d, J=6.36 Hz) 0.92 (3H, t, J=7.52 Hz) ^{13}C NMR (CDCl$_3$, 300 MHz) δ 175.74, 68.32, 60.70, 54.5, 22.8, 21.75, 14.56, 11.9

Ethyl 2(*S*)-allyl-3(*S*) hydroxybutanoate

^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.72 (1H, m) 5.04 (2H, dd, J_{cis} = 13.7 Hz, J_{gem} = 19.5 Hz, J_{vic} = 1.71 Hz) 4.15 (2H, q, J = 7.08 Hz) 3.9 (1H, m) 2.72 (1H, s, OH) 2.33-2.48 (3H, m) 1.25 (3H, t, J = 7.08 Hz) 1.21 (3H, d, J = 6.11 Hz) ^{13}C NMR (CDCl$_3$, 300 MHz) δ 175.0, 135.0, 117.4, 68.1, 60.8, 52.4, 33.86, 21.6, 14.5

Ethyl 2(*S*)-propargyl-3(*S*) hydroxybutanoate

^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.21 (2H, q, J=7.13 Hz) 4.11 (1H, m) 2.71 (1H, s, OH) 2.57 (3H, m) 2.03 (1H, dd, J=2.5, J=2.7 Hz) 1.28 (3H, t, J=7.13 Hz) 1.25 (3H, d, J=6.36 Hz) ^{13}C NMR (CDCl$_3$, 300 MHz) δ 173.76, 80.93, 70.58, 67.58, 61.28, 51.1, 21.3, 18.85, 14.49

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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May 2000

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